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The Journal of Biochemistry, Vol. 44, No. 10, 1957

ACID INACTIVATION OF TAKA-AMYLASE A

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(Received for publication, March 4, 1957)

In 1940, acid inactivation of taka-amylase and its reactivation by neutralisation with alkali were reported (*1*). The enzyme was not obtained as a crystalline state at that time, though considerably purified. The present author has studied more precisely on the condition of the acid inactivation by using crystalline and electrophoretically homogenious taka-amylase A (TAA). In the acid inactivation, a considerable amount of amino acid liberation was observed. Dynamic chemical properties of TAA was followed through the determination of liberated amino acids.

MATERIALS AND METHODS

Taka-amylase A—Crystalline TAA prepared from "Takadiastase Sankyo" was recrystallised three times from aqueous acetone (*2*).

Acid taka-protease—Purified acid taka-protease, prepared by the method of Amano *et al.* (3), was supplied by Dr. Isojima of the Department of Bacteriology Medical School, Osaka University.

Heat-treated-TAA (TAAh) was prepared by the following procedure: one volume of *M*/2 calcium acetate was added to 4 volumes of 5 per cent TAA solution for protection of its enzymatic activity, and pH was adjusted to 7 by adding saturated sodium bicarbonate solution. This solution was heated at 60° for ten minutes and then cooled in ice water. Adding Acetone the precipitated TAAh was redissolved in distilled water, and insoluble denatured protein was filtered off. A prolonged heating, more than ten minutes, will probably cause big loss of TAA.

The amylase activity was measured by modified Willstaetter-Schudel's method (*3*).

Sanger's DNFB method was applied for the analysis of amino acid and for the identification of N-terminal amino acid.

The concentration of TAA was estimated by determining protein-N by the micro Kjeldahl method.

EXPERIMENTS AND RESULTS

To the aqueous solution of TAA, 0.5 *M* acetic acid was added until pH 3.0, and incubated at 30°. The activity of the mixture was measured by occasional sampling and compared with its initial activity. The decrease in amylase activity proceeded rapidly at first, being followed by its gradual loss. This seemed to obey the first order reaction. This solution containing partially inactivated TAA, was neutralised by $NaHCO_3$, and the enzymatic activity was restored to some extent. This reversible inactivation occurred, also, by such other acids as hydrochloric

TABLE I

Inactivation and Reactivation of TAA

Inactivation			Reactivation*		Recovery** of activity (%)	Temperature
Acid used	pH	Remaining activity (%)	pH	Final activity (%)		
acetic	3.0	8	7.4	44	39	30°
HCl-citrate	3.7	5	7.0	34	31	24
phosphoric	2.7	5	7.0	38	35	24

* neutralized by $NaHCO_3$.

** (final activity—remaining activity) / (100—remaining activity).

TABLE II

*Inactivation and Reactivation of TAAh** (30°)

Exp. No.	Inactivation				Reactivation			Recovery of activity (%)
	Acid used	pH	Time (*min.*)	Remaining activity (%)	Neutralizing reagent	pH	Final activity (%)	
1.	acetic	3.2	40	32	$NaHCO_3$	7.0	80	71
			70	13			58	52
			120	4			42	40
2.	acetic	2.8	10	55	Na_2HPO_4	7.0	75	45
			40	11			47	40
			123	1			33	33
3.	phosphoric	2.8	10	32	$NaHCO_3$	7.0	100	100
			40	5			75	74
			60	3.5			71	70
4.	phosphoric	2.4	60	3	$NaHCO_3$	7.0	90	90
5.**	phosphoric	2.4	60	1.5	$NaHCO_3$	7.0	28	27

* The concentration of TAAh was 2.8 mg./ml.

** In Exp. 5, purified taka-protease was added.

and phosphoric acid. When the activity was lowered under 5 per cent original activity by the acid, its recovery was 30 per cent to 40 per cent

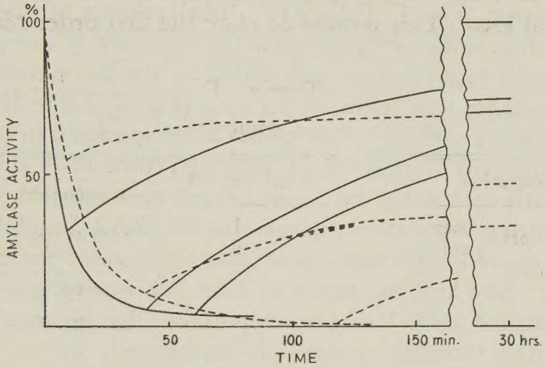


FIG. 1. Restoration of amylase activity

2.8 mg./ml. TAAh was incubated in phosphoric acid and acetic acid respectively (pH 2.8, 30°). These were neutralized by $NaHCO_3$ for former, by Na_2HPO_4 for the latter, to pH 7.0. (——), phosphoric acid inactivation; (– – –), acetic acid inactivation.

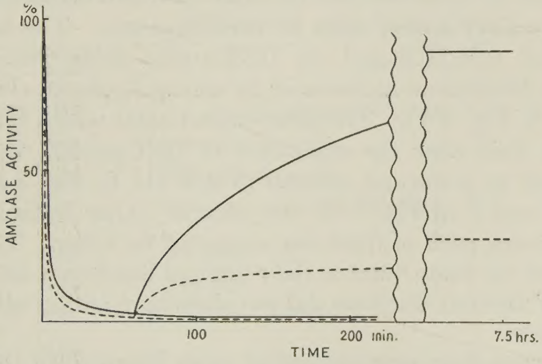


FIG. 2. Inhibition of restoration of amylase activity by acid taka-protease.

2.8 mg./ml. TAAh was incubated in phosphoric acid (pH 2.4, 30°) with (– – –) and without (——) acid Taka-protease. After 1 hour, by adding $NaHCO_3$, solution was neutralized to pH 7.0.

of the lost activity. The results of the experiments are shown in Table I.

Then TAAh was incubated under the same conditions, until the activity decreased below 5 per cent. Phosphoric acid inactivated TAAh twice as much in rate as acetic acid did, and the recovery of activity by neutralization was about 70 per cent in the former case, and 40 per cent in the latter case (Fig. 1, Table II). The presence of phosphate at the reactivation had nothing to do with the recovery of activity.

When acid taka-protease was added to the incubated solution, the recovery of activity was below 30 per cent (Fig. 2, Table II).

Under these conditions, free amino acids appeared in the incubating solution. Amounts of amino acids liberated from TAA and TAAh were compared with each other for their 0.5 per cent solution, pH of which was 3.4. During two days' incubation at 37°, TAA and TAAh liberated 7.6 and 5.1 μg. of amino acids, as leucine equivalent, per mg. of protein, respectively. The heat treatment of TAA depressed slightly the amount of liberated amino acids, losing much protein. In this treatment, specific amylase activity remained practically unchanged.

The liberated amino acid and the N-terminal amino acid of incubated TAA were determined. TAA was inactivated with HCl-citrate buffer of pH 3.7 at 24°, and after 3.5 hours the activity was 5 per cent of the initial one. NaOH added until pH 7.0, the activity was restored up to 31 per cent. The incubation mixture was then dinitrophenylated by usual method (*4*), and separated dinitrophenyl (DNP)-protein from DNP-amino acids by centrifugation. The former was hydrolysed with 6 *N* HCl and the DNP-amino acids obtained were determined as N-terminal amino acid by silicagel column chromatography (Table III A, Fig. 3 A). The DNP-amino acids which remained in the supernatant fluid after the separation of DNP-protein were also chromatographed by a silicagel column (Table III B, Fig. 3 B), and bands A, B, C, 2 and 3 in Fig. 3 B were eluted. After hydrolysis with 6 *N* HCl for 6 hours, each of them was extracted by ether. These ether extracts showed the same band as their original band on a silicagel column, and each of aqueous fractions did not show any amino acid on a paper chromatogram.

In order to determine the amino acids liberated in the initial stage of incubation of TAA, a short time inactivation was carried out. The solution (containing 2.8 mg. of TAAh per ml.) was brought to pH 2.8 by adding phosphoric acid and incubated for ten minutes at 30°. The activity was 32 per cent of the original one. Then it was neutralized and the greater portion of it was dinitrophenylated. As the N-terminal amino acid of protein fraction only alanine was found. And as the liberated amino acids, comparable amounts of serine, glycine and alanine and trace of threonine and/or glutamic acid were detected (Fig. 4]. After this neutralization TAAh restored its full activity (Table II).

TABLE III

(*A*) *N-terminal Amino Acids of Native and Incubated TAA*
Band No. corresponds to Fig. 3 A

Band No.	Native TAA (50.4 *mg.* DNP-TAA) DNP-amino acid recovered*		Incubated TAA (50.5 *mg.* DNP-TAA) DNP-amino acid recovered*	
	μM	M/M-TAA	μM	M/M-TAA
1.	trace		0.08	0.01
2.	trace		0.04	0.05
3.	trace		0.05	0.07
4. (Ala)	0.5	0.7	0.6	0.8
5.	—		0.03	0.04
6.	—		0.03	0.04

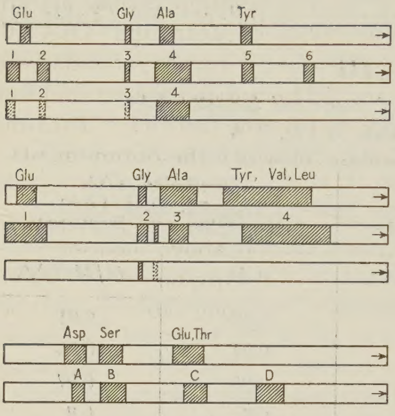
(*B*) *Amino Acids Liberated during Incubation of TAA* (*70 mg.*)
Band No. corresponds to Fig. 3 B

Band No.	DNP-amino acids liberated*	
	μM	M/M-TAA
1.	1.87	1.4—It was separated further to A, B, C and D.
2.	0.45	0.3—Gly. Fraction
3.	0.94	0.6—Ala. Fraction
4.	5.77	4.0—Leu. and Val. Fraction
A.	0.35	0.4—Asp.
B.	0.30	0.4—Ser.
C.	0.41	0.5—Glu. Fraction
D.	0.10	

* These were determined by colorimetry of DNP-amino acid.

DISCUSSION

During the acid inactivation, TAA liberates a considerable amount of some definite kinds of free amino acids, that is, liberating serine, glycine and alanine during the initial stage of inactivation. These



A. N-terminal amino acids of incubated TAA. (5BC-5AW).

standard

hydrolysate of DNP-TAA-incubated

hydrolysate of DNP-TAA-native

B. Liberated amino acids from incubated TAA. (5BC-5AW).

standard

from the incubated solution

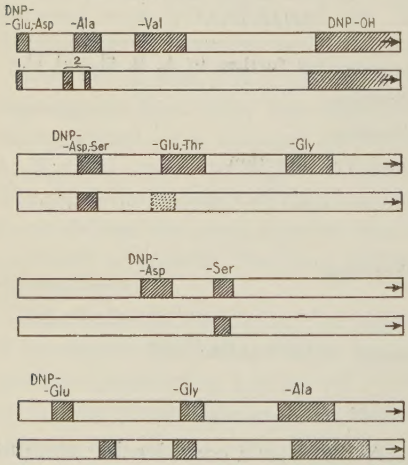
from TAA solution before incubation

Band No. 1 in above column. (20BC-10AW).

standard

FIG. 3. N-terminal and liberated amino acids of TAA after incubation.

The notation of, solvent system is as follows: B, C, A and W are butanol, chloroform, gracial acetic acid and water, respectively, and the figures before B and A are the volume percentage of B to B+C and A to A+W, respectively.



A. Liberated amino acids from incubated TAAh (2BC-2AW)

standard

B. Band No. 1 in column A. (10BC-10AW)

standard

C. Band No. 1 in column A. (Ethyl acetate-2AW)

standard

D. Band No. 2 in column A. (5BC-5AW)

standard

FIG. 4. Liberated amino acids from TAAh at initial stage of incubation.

amino acids are the C-terminal amino acid of TAA (*5*). The N-terminal amino acid of remaining protein after incubation is only alanine, which has been estimated to be N-terminal of untreated TAA (6). This feature of the docomposition suggests that a carboxypeptidase-like enzyme will contaminate TAA and act to liberate the amino acid from C-terminal. This possibility was supported by the fact that "Taka-diastase Sankyo" contains acid taka-protease, of which the optimum pH is near 3, and that the specificity of this protease is carboxypeptidase-like (*7*). The experiment using acid taka-protease described in this paper (Fig. 2) is also consistent with the interpretation above mentioned.

On the other hand, crystalline TAA is electrophoretically homogenious. The heat treatment was not effective enough to stop the liberation of amino acids, however, TAAh has not a detectable proteolytic activity against horse serum and haemoglobin which were good substrates of the acid taka-protease. From these facts, the contaminating acid taka-protease, if it were contained in TAAh, may be partly responsible for the amino acid liberation, and it is probable that the autodegradation is also included in this phenomenon. This anticipation was supported by the experiment of the amino acid liberation in neutral pH regions (*8*), where acid taka-protease is completely inactive.

The inactivation of TAAh by phosphoric acid is more rapid than by acetic acid in the same pH, and after the same inactivation time the restoration of the activity in the former case is much greater than in the latter. They seem to have different mechanism of inactivation.

SUMMARY

Crystalline taka-amylase A was reversibly inactivated by acid and restored its activity by neutralisation. In the inactivated state, a small quantity of free amino acids seems to be liberated from C-terminal.

The author wishes to express his gratitude to Professor S. Akabori for his kind guidance, and to Mr. T. Ikenaka for his helpful advice through this investigation, and also to Snakyo Co. Ltd. for their kind supply of "Takadiastase Sankyo."

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The Journal of Biochemistry, Vol. 44, No. 10, 1957

ÜBER DIE PRÄPARATIVE DARSTELLUNG VON *p*-FLUORPHENYLALANIN

VON TÔRU OKUDA UND SUSUMU TATSUMI

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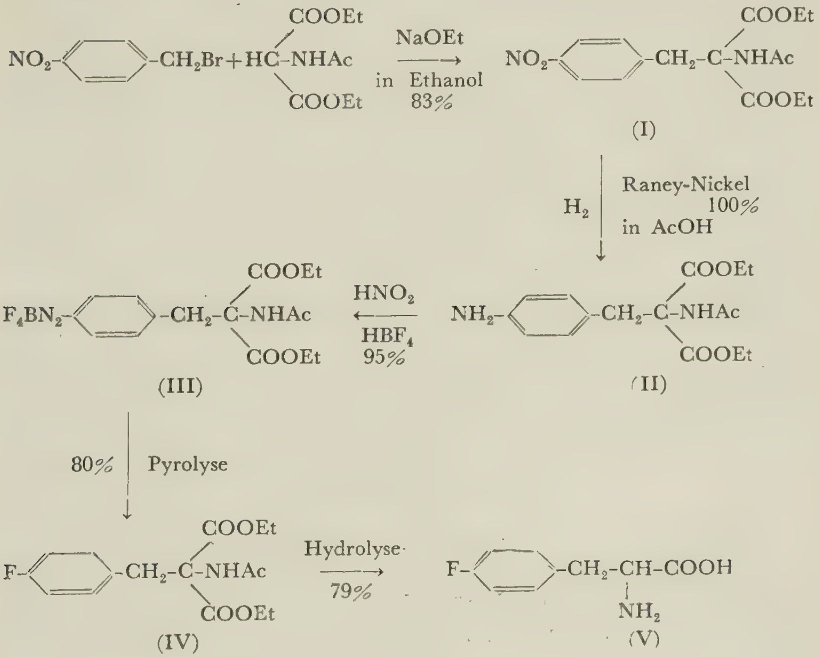
(Der Schriftleitung zugegangen am 24 April, 1957)

Für die Gewinnung des Phenylalanin-Antagonisten *p*-Fluorphenylalanin, das wegen der nachher klargestellten antimetabolischen Bedeutung immer an Interesse von vielen Forschern gewonnen hat, sind einige Darstellungsmethoden bekannt geworden.

Die erste Synthese dieser Aminosäure von G. Schiemann und W. Roselius (*1*) geht von *p*-Fluorbenzaldehyd aus, das nach der Umsetzung mit Hippursäure durch nachfolgende reduktive Hydrolyse von



Azlacton ins Endprodukt übergeht. Der Nachteil dieser Synthese liegt in der Schwerzugänglichkeit des als Ausgangsmaterial dienenden *p*-Fluorbenzaldehyds. Die andere Gruppe von Verfahren zur Darstellung dieses Antagonisten geht von N-substituierten Aminomalonsäureestern oder von Acetamino-cyanessigester aus, in deren reaktionsfähiges Kohlenstoffatom der *p*-Fluorbenzyl-Rest durch *p*-Fluorbenzylchlorid eingeführt wird. Auf diese Weise haben E. L. Bennet und C. Nieman (*2*) aus Acetamino-malonsäure-diäthylester und Acetamino-cyanessigsäure-äthylester, und E. D. Bergmann (*3*) aus Phthalimino-malonsäure-diäthylester jene Aminosäure in mässiger Ausbeute dargestellt.

Die vorliegende Arbeit zeigt eine neue Darstellungsweise des *p*-Fluorphenylalanins aus leicht zugänglichem *p*-Nitrobenzylbromid und Acetamino-malonsäure-diäthylester, welche nach dem Reaktionsschema gelungen ist.

Von vier obigen substituierten Malonesterderivaten sind schon vor einigen Jahren Nitro- (I) und Amino-Verbindung (II) in Form des Hydrochlorids durch J. H. Burckhalter und V. C. Stephens (*4*) dargestellt worden. Der Nitro-Körper (I) lässt sich durch katalytische Hydrierung in essigsaurer Lösung mit Raney-Nickel bei 70–80° in quantitativer Rohausbeute in entsprechendes freie Amino-Derivat (II) überführen. Hierbei ist berichtenswert, daß, wenn die Reduktion in alkoholischer statt essigsaurer Lösung durchgeführt wird, die erhaltene Kristalle des Amino-Derivats selbst nach vielmaligem Umkristallisieren noch durch anhaftende gefärbte Beimengungen verklebt sind. Dies ist vermutlich durch Alkylierung der Aminogruppe bedingt, welche von dem als Lösungsmittel verwendeten Alkohol bei solcher katalytischen Druckhydrierung mit Raney-Nickel in erhöhter Temperatur bewirkt wird (*4*).

Die Einführung des Fluors in den Benzolkern gelingt auch hier bequem nach der gewöhnlichen Schiemann-Methode (*6*) in guter Ausbeute. Durch Hydrolyse des *p*-Fluorbenzyl-malonesters (IV) mit Salzsäure erhält man DL-*p*-Fluorphenyl-β-alanin (V) in einer Totalausbeute von 50 pro zent (bez. auf Benzylbromid).

BESCHREIBUNG DER VERSUCHE

p-Nitrobenzylbromid wurde nach G. H. Coleman und G. F. Honeywell (*7*) dargestellt.

p-Nitrobenzyl-acetamino-malonsäure-diäthylester (I): Der Ester wurde nach dem Burckalterschen allgemeinen Verfahren (*5*) dargestellt. 3 g.

(0.13 Atom) Natrium wurde in 100 ml. absoluten Alkohol in einem Rundkolben von 300 ml. Inhalt gelöst. Zu dieser noch heissen Lösung werden 28.2 g. (0.13 *M*) Actamino-malonsäure-diäthylester unter Feuchtigkeitsausschluss gegeben und dann auf einmal eine Lösung von 28.0 g. (0.13 *M*) *p*-Nitrobenzylbromid in 70 ml. heissen absoluten. Alkohols zugegeben, wobei *p*-Nitrobenzyl-acetamino-malonsäure-diäthylester sofort in kaum gefärbten Kristallen sich abscheidet, die Lösung wird 1 Stunde unter Rückfluss gekocht. Das breiartige Reaktionsgemisch wird in 1.5 Liter Eiswasser gegossen, um das entstandene Natriumbromid auszulösen und die Abscheidung des Nitroesters zu vollenden. Filtration und Auswaschen mit Wasser geben 38 g. (83-prozent d. Th.) *p*-Nitrobenzyl-acetamino-malonsäure-diäthylester vom Schmerzpunkt 186–189°. Blättchen nach Umkristallisieren aus Eisessig-Wasser oder Alkohol. Schmelzpunkt 188–189° (Lit. Schmelzpunkt, 193–194° (5)).

$C_{16}H_{20}O_7N_2$ (352)
Ber.; C, 54.55 H, 5.68 N, 7.95
Gef.; C, 54.18 H, 5.72 N, 8.02

p-Aminobenzyl-acetamino-malonsäure-diäthylester (II): Eine Suspension von 15.8 g. (0.045 *M*) rohen *p*-Nitrobenzyl-acetamino-malonsäure-diäthylesters (I) (Schmelzpunkt, 186–189°) im 150 ml. Eisessig wird bei Gegenwart von aus 3 g. Nickel-Aluminium-Legierung dargestellten Raney-Nickel in einem Hochdruckautoklaven unter mechanischer Schütterung bei 70–80° und 70 atm. Wasserstoffdruck hydriert. Innerhalb von 2 Stunden hat die Lösung die berechnete Menge Wasserstoff aufgenommen. Nach dem Einengen der vom Katalysator abfiltrierten Lösung bleibt ein gelblich grünes zähflüssiges Öl, das nach Zusatz von 70 ml. Wasser und Neutralisierung mit festem Natriumcarbonat beim Reiben mit dem Glasstab sofort kristallisiert. Gelbliche Kristalle vom Aminoderivat (II) betragen 14 g. Schmelzpunkt, 127–129°. Aus Mutterlauge werden weitere Kristalle (0.5 g.) gewonnen. Totalausbeute 14.5 g. (quantitativ). *p*-Aminobenzyl-acetamino-malonsäure-diäthylester (II) schmilzt nach Umkristallisieren aus 30-prozent heissen wässrigen Alkohol bei 133°. Blättchen. Ausbeute 10.5 g.

$C_{16}H_{22}O_5N_2$ (322)
Ber.; C, 59.63 H, 6.83 N, 8.70
Gef.; C, 59.69 H, 6.68 N, 9.04

p-(2,2-Dicarbäthoxy-2-acetaminoäthyl)-phenyl-diazonium-borfluorid (III):

Zu 30 ml. Wasser lässt man in einem 200 ml. Becherglass unter gutem Rühren 8 g. konzentrierter Salzsäure (0.08 *M*) laufen und in diese warme verdünnte Säure werden 10.5 g. (0.032 *M*) feingepulverten *p*-Aminobenzyl-acetamino-malonsäure-diäthylesters (II) eingeworfen. Dann lässt man zu der mit Kältemischung gekühlten Chloridlösung, aus der sich das schwer lösliche Salz teilweise ausscheidet, aus einem Tropftrichter allmählich unter tüchtiger Umrührung die Lösung von 2.5 g. Natriumnitrit (0.036 *M*) in 10 ml. Wasser fliessen. Überschüssiger Zusatz des Nitrits (wird vermieden durch) Prüfung mit Kaliumjodid-Stärkepapier. Nach beendeter Diazotierung geht das Chlorid vollständig in Lösung. Die Lösung wird mit Borflußsäure (aus 5.5 g. 50-prozent Flußsäure und 2.1 g. Borsäure) versetzt, wobei sofort gelbliches Diazonium-borfluorid ausfällt. Der ausgeschiedene Kristallkuchen wird abgesaugt, mit wenig Wasser gewaschen und getrocknet. Die Ausbeute an Borfluorid (III) beträgt 13 g. (95 prozent d. Th.). Der Zersetzungspunkt liegt bei 123°.

p-Fluorbenzyl-acetamino-malonsäure-diäthylester (IV) : 13 g. (0.0309 *M*) scharf getrockneten *p*-(2, 2 - Dicarbäthoxy- 2 - acetaminoäthyl) - phenyl-diazonium-borfluorids (III) werden in einem 100 ml.-Rundkolben unter Evakuieren zuerst über freier Flamme erwärmt. Die Zersetzung geht glatt vor sich unter heftiger Aufschäumung (Stickstoff und Bortrifluorid). Nach Umkristallisierung des brauen Rückstands aus siedendem wässrigen Alkohol—im voraus muss die Lösung mit Natriumcarbonat neutralisiert werden—und nach eintägigem Stehenlassen im Eisschrank erhält man weisse Kristalle von *p*-Fluorbenzyl-acetamino-malonsäure-diäthylester (IV) vom Schmelzpunkt 139–141°. (Lit. Schmelzpunkt, 144° (*2*)). Ausbeute 8 g. (80 pro zent d. Th.).

$C_{16}H_{20}O_5N_4F$ (325)
Ber.; C, 59.08 H, 6.15 N, 4.13
Gef.; C, 59.16 H, 6.30 N, 4.33

p-Fluor-β-phenylalanin (V) : 47 g. (0.144 *M*) *p*-Fluorbenzyl-ace-t amino-malonsäure-diäthylester (IV) werden mit 300 ml. 6 *N* Salzsäure 4 Stunden lang hydrolisiert. Nach dem Verdampfen der überschüssigen Salzsäure wird das ausgeschiedene Aminosäure-Hydrochlorid in wässrigem Methanol gelöst, mit Kohle entfärbt, sodann mit Ammoniakwasser neutralisiert und abgekühlt. Weisse Kristalle von *p*-Fluor-β-phenylalanin (V) beträgt 21 g. (79 prozent d. Th.). Es zersetzt sich bei 260–265°.

$C_9H_{10}O_2NF$ (183)
Ber.; C, 59.01 H, 5.46 N, 7.65
Gef.; C, 58.77 H, 5.45 N, 7.51

ZUSAMMENFASSUNG

Der Phenylalanin-Antagonist *p*-Fluorphenylalanin läßt sich von Acetamino-malonsäure-diäthylester und *p*-Nitrobenzylbromid aus über *p*-Nitro-, *p*-Aminobenzyl-acetamino-malonsäure-diäthylester, das Diazonium-borfluorid und *p*-Fluorbenzyl-acetamino-malonsäure-diäthylester in 50-prozent Ausbeute darstellen.

SUMMARY

p-Fluorophenylalanine which is active as phenylalanine antagonist has been synthesized from diethyl acetamidomalonate and *p*-nitrobenzyl bromide *via* diethyl *p*-nitro-, *p*-aminobenzyl-acetamidomalonate, the diazoninium fluoroborate and diethyl *p*-fluorobenzyl-acetamidomalonate in an over-all yield of 50 per cent.

Herrn Prof. Dr. S. Akabori und Herrn Y. Izumi sprechen wir für wertvolle Ratschläge unseren aufrichtigen Dank aus.

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The Journal of Biochemistry, Vol. 44, No. 10, 1957

DENATURATION AND INACTIVATION OF ENZYME PROTEINS

VIII. THERMODYNAMIC ASPECTS OF THE DENATURATION OF CRYSTALLINE α-AMYLASE OF BACILLUS SUBTILIS*

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At present, it is believed that the denaturation of globular protein begins with degradation of the secondary structure of the protein molecule (*1*). Okunuki *et al.* (*2*) have recently reported a method by which the denatured protein and the native one can be separately quantified in their mixture by using a bacterial proteinase prepared from culture medium of *Bacillus subtilis* (*3*). The method seems to give a criterion on the degradation of the secondary structure of the protein molecule. In these experiments, the degradation of crystalline α-amylase of *B. subtilis* was investigated under various conditions comparing the denaturation by proteinase digestion method with the inactivation by Fuwa's method (*4*). Some thermodynamic data of the denaturation under these conditions and some behaviours during its degradation processes will be described, in order to contribute to the information on the nature of denaturation of enzyme proteins.

EXPERIMENTAL

Preparation of Enzymes

α-Amylase—α-Amylase of *B. subtilis* (*3*) was recrystallized four times from 0.01 *M* calcium acetate solution at pH 6.0 in the presence of 20 per cent acetone. When the crystals prepared in this manner were used, the proteinase digestion and inactivation proceeded to the same extent.

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* A preliminary report of this work was presented at the Meeting of the 30th Anniversary of the Foundation of the Japanese Biochemical Society held in Tokyo, in November, 1955.

Bacterial Proteinase—The lyophilized crystals of proteinase of *B. subtilis* (*3*) were dissolved in 0.2 *M* borate buffer solution (pH 7.6) on use.

Measurements of Inactivation and Proteinase Digestion

Inactivation—0.5 ml. of the reaction mixture for denaturation was periodically pipetted out and added to 225 ml. of 0.01 *M* NaCl containing 0.002 *M* Na_2HPO_4. Then, 1.0 ml. of the mixture was added to 5.0 ml. of 1.0 per cent soluble starch dissolved in 0.02 *M* phosphate buffer solution (pH 6.5), and incubated at 30°. After five minutes incubation, 1.0 ml. of the mixture was added to 3.0 ml. of Somogyi's new copper-carbonate-tartrate reagent (*4*) and the mixture was heated for 30 minutes in a boiling water bath. It was immediately chilled by ice water to a room temperature and mixed with 3.0 ml. of Nelson's arsenomolybdate reagent (*4*). The mixture was diluted with distilled water to 50 ml. in a volumetric flask and its optical density was measured at 500 mμ by a Shimadzu photoelectric spectrophotometer (type QB-50). The degree of inactivation was expressed by the following equation:

$$\text{Ratio of inactivation }(\%)=\frac{E_{500\ \text{initial}}-E_{500\ t}}{E_{500\ \text{initial}}-E_{500\ \text{final}}}\times 100,$$

where $E_{500\ \text{initial}}$ is the optical density at 500 mμ for initial amylase solution, $E_{500\ t}$ that for the solution of time interval *t*, and $E_{500\ \text{final}}$ that for the solution of completely inactivated amylase.

Proteinase Digestion—Into 1.0 ml. of 0.04 per cent proteinase solution previously preserved at 30°, 0.5 ml. of the α-amylase solution for denaturation was added and incubated at 30° for 10 minutes. Then 5.0 ml. of 0.6 *M* trichloroacetic acid was added to the incubated mixture and filtered. The optical density of the filtrate was measured at 280 mμ by the photoelectric spectrophotometer. The degree of denaturation was expressed by the following equation:

$$\text{Ratio of denaturation }(\%)=\frac{E_{280\ t}-E_{280\ \text{initial}}}{E_{280\ \text{final}}-E_{280\ \text{initial}}}\times 100,$$

where $E_{280\ t}$ is the optical density at 280 mμ for the amylase solution of time interval *t*, $E_{280\ \text{initial}}$ that for the initial solution, and $E_{280\ \text{final}}$ that for completely denatured amylase. The ratio measured by the Folin colour reaction (*3*) was the same value as that measured by optical density at 280 mμ.

RESULTS

Urea-, heat-, and acid-denaturation of α-amylase were investigated. Each of the denaturations was carried out both in the absence and presence of Ca^{++}, since α-amylase has been known to be considerably protected by the ion from denaturation (*3*). The denaturation processes are shown in Fig. 1 and Table I. The denaturation follows first-order kinetics with regard to concentration of the amylase as indicated in Fig. 2. The specific velocity constant of the denaturation can be calculated according to the following equation:

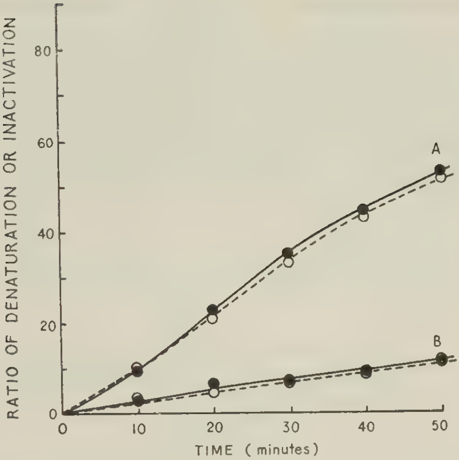

FIG. 1. Processes of denaturation and inactivation of α-amylase in 6.0 M urea solution (pH 8.0) at 50°.

(A) In the absence of, and (B) in the presence of 0.05 M $CaCl_2$. In the case of (A) 0.02 M Na_2HPO_4 was added but not in the case of (B). ●—● Denaturation ○---○ Inactivation. Concentration of α-amylase is 0.32 per cent in each case.

TABLE I

An Example in Measurement of Ratios of Inactivation and Denaturation

The same values are plotted in Fig. 1

Time (*min.*)	At 500 $m\mu$			At 280 $m\mu$		
	E	ΔE	%	E	ΔE	%
0	0.546	0.000	0.0	0.239	0.000	0.0
10	0.488	0.058	10.6	0.306	0.067	9.7
20	0.437	0.109	20.0	0.422	0.183	26.9
30	0.359	0.187	34.2	0.515	0.276	39.8
40	0.311	0.235	43.0	0.546	0.307	45.1
50	0.259	0.287	52.5	0.609	0.370	54.3
∞	0.000*	0.546	100.0	0.920**	0.681	100.0

* A totally denatured sample was used as the control.

** This sample was boiled at 100° for 10 minutes.

$$k \cdot \Delta t = 2.3 \cdot \log (a/a-x) \qquad (1),$$

where a is the initial amount of native protein, x the amount of denatured protein for time interval Δt, and k the velocity constant.

The processes of denaturation were observed at various temperature. In Fig. 3, logarithm of k is plotted against reciprocal of the absolute temperature. Applying the Arrhenius equation:

$$\log k = -\frac{E}{2.3 \cdot RT} + \text{constant} \qquad (2),$$

where k stands for the specific reaction rate constant, E for the activation energy, R for the gas constant, and T for the absolute temperature, the activation energy of the denaturation of amylase is obtained from the gradient of the curve in Fig. 3 and 4. Other thermodynamic values, ΔH^* (the enthalpy of activation), ΔG^* (the free energy of activation), and ΔS^* (the entropy of activation) are calculated by the following equations derived from absolute reaction rate theory (*5*):

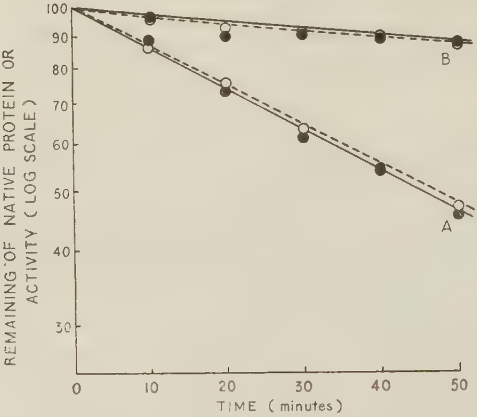

FIG. 2. Processes of denaturation and inactivation of α-amylase in 6.0 *M* urea solution (pH 8.0) at 50°.

Curves were plotted on semi-logarithmic section paper. The other experimental conditions were the same as those described in Fig. 1. ●—● Denaturation ○---○ Inactivation.

$$\Delta H^* = E - RT \qquad (3)$$

$$\Delta G^* = 4.58\, T\, (10.318 + \log T - \log k) \qquad (4)$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \qquad (5)$$

These data are presented in Table II.

The denaturation processes in the presence of urea are shown in Fig. 1, and that of heat- and acid-denaturation in Fig. 5.

The k at various concentrations of urea is also calculated and the $\log k - 1/T$ curves are shown in Fig. 6, and thermodynamic data in Table III.

In Fig. 7, logarithm of half time for the urea-denaturation is plotted against logarithm of activity of urea. The activity (*i.e.*, true effective concentration) of urea is calculated using data of Scatchard *et al.* (*6*) and Gucker *et al.* (*7*).

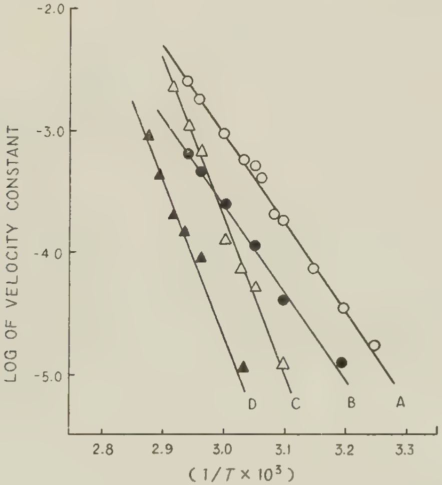

FIG. 3. Temperature dependence of velocity constants of denaturations under various conditions.

(A) 6.0 *M* Urea-denaturation in the absence of $CaCl_2$. (B) 6.0 *M* Urea-denaturation in the presence of 0.05 *M* $CaCl_2$. (C) Heat-denaturation in the absence of $CaCl_2$. (D) Heat-denaturation in the presence of 0.05 *M* $CaCl_2$. In the case of (A) and (C), 0.02 *M* Na_2HPO_4 was present.

As described above, Ca^{++} considerably protects α-amylase from its denaturation. However protection in heat-denaturation seems to be different from protection in urea-denaturation, since in the absence of Na_2HPO_4, Ca^{++} does not protect the amylase recrystallized in 0.01 *M* calcium acetate solution from heat-denaturation, while it protects the enzyme from urea-denaturation (Fig. 8). However, even in the urea-

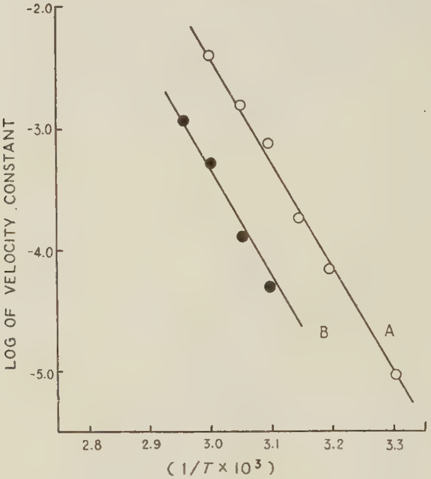

Fig. 4. Temperature dependence of velocity constants in acid-denaturation in 0.1 *M* acetate buffer solution (pH 4.5).

(A) In the absence of $CaCl_2$, (B) in the presence of 0.05 *M* $CaCl_2$.

TABLE II

Some Thermodynamic Data of Denaturations of Bacterial α-Amylase in Several Different Conditions

Conditions of denaturation					Thermodynamic data				
Kind of denaturation	Na_2HPO_4	$CaCl_2$	pH	Temp.	k (*sec.*$^{-1}$)	E (*kcal.*)	ΔH^* (*kcal.*)	ΔG^* (*kcal.*)	ΔS^* (*e.u.*)
6 *M* urea[a)]	0.02 *M*	—	8.0	50	1.73×10^{-4}	33.4	32.8	24.6	25.4
6 *M* urea	—	0.05 *M*	8.0	50	3.95×10^{-5}	33.4	32.8	25.5	22.6
Heat	0.02 *M*	—	8.0	50	1.12×10^{-5}	68.3	67.6	26.4	127.5
Heat	—	0.05 *M*	8.0	50	(4.18×10^{-7})[b)]	68.3	67.6	(28.4)	121.5
Acid[c)]	—	—	4.5	50	7.07×10^{-4}	40.2	39.5	23.7	49.0
Acid	—	0.05 *M*	4.5	50	5.10×10^{-5}	40.2	39.5	25.4	43.7

a) Concentration of urea is presented in moles per 1000 ml. of solution.

b) Obtained by extrapolation using the log k—$1/T$ curve.

c) 0.1 *M* acetate buffer solution was used to maintain the pH at 4.5.

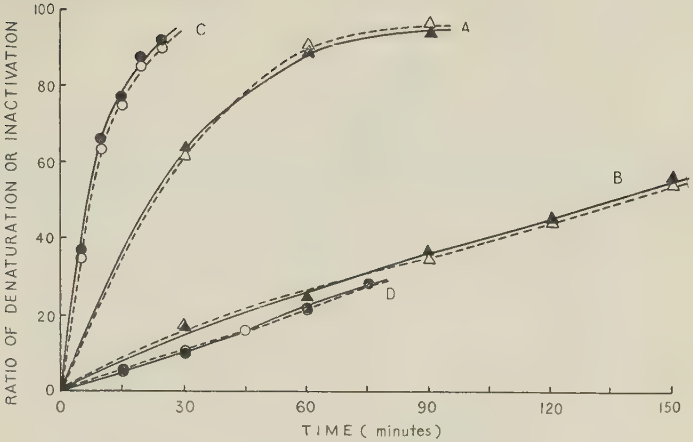

FIG. 5. Processes of heat- and acid-denaturation of α-amylase.

(A) Heat-denaturation at pH 8.0 in the absence of $CaCl_2$ and in the presence of 0.02 *M* Na_2HPO_4. (B) Heat-denaturation at pH 8.0 in the presence of 0.05 *M* $CaCl_2$ without Na_2HPO_4. (C) Acid-denaturation in 0.1 *M* acetate buffer solution (pH 4.5) in the absence of $CaCl_2$ and Na_2HPO_4. (D) Acid-denaturation in 0.1 *M* acetate buffer solution (pH 4.5) in the presence of 0.05 *M* $CaCl_2$ without Na_2HPO_4. Temperatures were 65° in (A) and (B), and 55° in (C) and (D).

—— Denaturation, – – – Inactivation. Concentration of α-amylase is 0.32 per cent in each case.

TABLE III

Some Thermodynamic Data of Urea-Denaturations of Bacterial α-Amylase in Varied Urea Concentrations

Conditions of denaturation					Thermodynamic data				
Concentration of urea *(mol.)*	Na_2HPO_4	$CaCl_2$	pH	Temp.	k *(sec.$^{-1}$)*	E *(kcal.)*	ΔH^* *(kcal.)*	ΔG^* *(kcal.)*	ΔS^* *(e.u.)*
1.0 *M*	0.02 *M*	—	8.0	50	3.80×10^{-5}	48.1	47.5	25.5	68.1
3.0 *M*	0.02 *M*	—	8.0	50	1.63×10^{-4}	38.2	37.6	24.6	40.3
6.0 *M*	0.02 *M*	—	8.0	50	1.73×10^{-4}	33.4	32.8	24.6	25.4

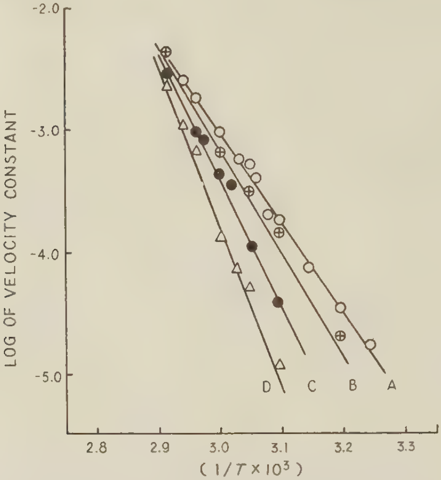

FIG. 6. Temperature dependence of velocity constants in various concentrations of urea in the presence of 0.02 *M* Na_2HPO_4 at pH 8.0. Concentrations of urea are (A) 6.0 *M*, (B) 3.0 *M*, (C) 1.0 *M*, and (D) nil.

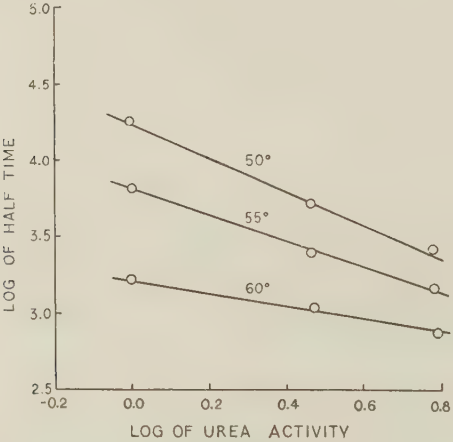

FIG. 7. Effect of urea activities on the half times of denaturation at different temperatures in the presence of 0.02 *M* Na_2HPO_4 (pH 8.0).

The gradients of the curves are ca. −1.1, −0.9, and −0.4 for 50°, 55°, and 60°, respectively.

denaturation, the excessive increase of Ca^{++} added does not promote the protection and appears to increase somewhat the degradation.

DISCUSSION

In all cases, the proteinase digestion and inactivation of α-amylase proceeded to the same extent when the crystals prepared under the conditions described above were used. Though it has been thought in general that only the active center is attacked in the inactivation of enzyme by acid (*8*), the result shown in Fig. 5 leads us to the consideration that not only the active center but also the whole globular configuration of α-amylase is attacked in acid-denaturation, because the enzyme inactivated by acid can be digested easily by the proteinase, as well as the enzyme denatured by urea or heat.

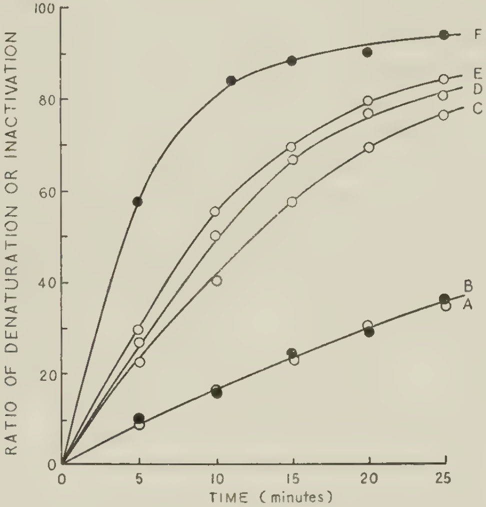

FIG. 8. Protection of α-amylase from its denaturation by $CaCl_2$ at 70° under various conditions.

(A) In the presence of 0.05 *M* $CaCl_2$ without Na_2HPO_4. (B) In the absence of $CaCl_2$ and Na_2HPO_4. (C), (D), (E), (F) 6.0 *M* urea-denaturation without Na_2HPO_4, in the presence of $CaCl_2$ in (C) 0.05 *M*, (D) 0.1 *M*, (E) 0.2 *M* and (F) nil, at pH 8.0, respectively. Concentration of α-amylase is 0.32 per cent in each case.

In each of three kinds of denaturation procedures, *i.e.*, urea-, heat-, and acid-denaturation, Ca^{++} markedly protected α-amylase from denaturation, but the activation energy for a certain denaturing agent was constant regardless of the ion. Therefore, it seems that the mechanism of denaturation is the same whether the ion is present or not. A similar result has been obtained by Gorini and Felix with heat-denaturation of lysozyme; it is protected by Mn^{++} from denaturation, but the activation energy of denaturation in the presence of Mn^{++} does not differ from that in the absence of this ion (*9*).

On the other hand, the activation energy of heat-denaturation is twice as much as that of urea-denaturation. It can not be concluded only from this result that the number of hydrogen bonds broken during heat-denaturation is twice as many as that during urea-denaturation, because urea molecule also participates in the rupture of hydrogen bonds in the latter case, even if the differences of activation energies of both denaturations are caused only by differences in the number of hydrogen bonds broken.

According to Simpson and Kauzmann (*10*), the urea-denaturation of ovalubumin occurs by the following equation:

$$PU_{\bar{n}}+n^{*}U \rightarrow D \qquad (6),$$

where P is native protein, U urea, n the mean number of urea molecule bound by the native protein, n* the apparent order of the reaction with respect to urea, and D the denatured protein. They have shown that n* can be obtained by plotting logarithm of half time for the denaturation ($t_{\frac{1}{2}}$) against logarithm of the activity of urea:

$$-\log t_{\frac{1}{2}}=\log\frac{k\cdot\gamma_{p}}{(\log 2)\ \gamma^{*}}+n^{*}\cdot\log U \qquad (7),$$

where γ_p and γ^* stand for activity coefficient of native protein and activated complex, respectively, and k for the velocity constant of the denaturation. The first term on the right hand side in equation (*7*) must vary with urea concentration, because k is different in different concentration of urea even if temperature is constant. However, actually both in the experiment of Simpson and Kauzmann and in the present one, $\log t_{\frac{1}{2}}-\log U$ curves became linear, so it may be right that n* should be obtained by equation (*7*). From Fig. 7, the values of n* in the denaturation of α-amylase are found to be 1.1, 0.9, and 0.4 at 50°, 55°, and 60°, respectively.

Simpson and Kauzmann (*10*) derived the following equation:

$$\Delta H^* = \Delta H_0^* - n^* (H+h) - \bar{n}h \qquad (8),$$

where ΔH_0^* stands for the enthalpy of activation in the absence of urea, H for the heat of adsorption of urea on protein, and h for the reduction of the enthalpy of activation by a urea molecule adsorbed on protein at normal state. They have obtained 10 kcal. for $H+h$ of ovalbumin. If the value can be applied to the case of α-amylase, as n^* is a unity at 50°, $\bar{n}h$ must become 10, 20, and 25 kcal. for 1, 3 and 6 molar concentration of urea, respectively, at this temperature. As h can be supposed to be constant through all concentrations of urea, $\bar{n}$ may vary with the concentration of urea. However they said that 10 kcal. for $H+h$ seemed to be too large.

That α-amylase can be protected by Ca^{++} to a great degree from denaturation has already been described. However, in the absence of phosphate salt, the behaviour of α-amylase was quite different from that in its presence as shown in Fig. 8. α-Amylase appears not to require so much Ca^{++} for protection from heat-denaturation as for urea-denaturation, since the enzyme was recrystallized in 0.01 *M* Ca^{++} solution. It has been known that urea combines with $CaCl_2$ to form a complex, $CaCl_2\ 4(NH_2)_2CO$ (*11*). Therefore, if the protection of Ca^{++} appears in the urea-denaturation because Ca^{++} is combined with urea as such a complex, the more Ca^{++} is added, the higher will be the extent to which α-amylase is protected from denaturation. In the presence of a concentration of $CaCl_2$ over a certain limit, however, the denaturation was rather accelerated as shown in Fig. 8. These facts lead us to the conclusion that the urea-denaturation differs from heat-denaturation in its mechanism. If the mechanisms of both denaturations are quite different from each other, it may be difficult to calculate the enthalpy of activation for urea-denaturation from that for heat-denaturation.

SUMMARY

1. The denaturation of a crystalline bacterial α-amylase under several conditions was investigated and thermodynamic data were calculated.

2. The degree of proteinase digestion of α-amylase recrystallized in the method described and that of its inactivation were in good agreement.

3. Ca^{++} protected α-amylase from urea-denaturation, but not from heat-denaturation in the absence of Na_2HPO_4. The activation energy of the denaturation was of the same value in the presence or absence of Ca^{++} in the urea-, heat-, and acid-denaturation.

4. The activation energy of heat-denaturation was over twice as much as that of urea-denaturation.

5. From the difference of protective action of Ca^{++} on the denaturation is different from that of heat-denaturation.

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The Journal of Biochemistry, Vol. 44, No. 10, 1957

STUDIES ON THE ACID DIAZOREACTION AND 5- OR 7-HYDROXYINDOLE DERIVATIVES

OXIDATION OF THE BENZENE MOIETY OF INDOLE-LACTIC ACID, -PROPIONIC ACID AND -ETHYLAMINE *etc.* BY LIVER EXTRACT

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(Received for publication, May 10, 1957)

In a previous paper (*1*), it was reported that the positive acid diazoreaction could be given only by 7-hydroxyindole derivatives. Prior to this, the same reaction had also been reported by Stowe (*2*), who demonstrated it by paperchromatography that indole acetic acid, 5- or 7-hydroxyindole compounds gave this reaction. The following experiments were undertaken to clarify the cause of this discrepancy between the two groups of authors. In order to avoid the nitrous acid reaction on indole nucleous, diazobenzonium sulfonic acid was used instead of the diazoreagents in some of the experiments.

In the present paper the previous data were reconfirmed, together with evidence for the oxidation of some indole compounds at the 5- or 7-position of benzene moiety by liver extract.

EXPERIMENTAL AND RESULTS

I. Specificity of the Acid Diazoreaction

Materials and Methods:

(*1*) 5-Methylindole and indole pyruvic acid were synthesized according to the methods described by Robson (*3*) and Ellinger- Matsuoka (*4*), respectively.

7-Hydroxyindole acetic acid and 7-hydroxytryptophan were furnished by Dr. T. Sakan and Dr. O. Hayaishi, respectively.

(*2*) *Diazobenzonium Sulfonic Acid*—5 g. of sulfanilic acid dissolved in 50 ml. of hydrochloric acid (*Sp. Gr.* 1.19) was diluted to 1 liter by addition of distilled water. To the solution was added 20 ml. of 0.5 per cent $NaNO_2$ and mixed well. The mixture was evaporated *in vacuo*, the resulting crystal was collected and recrystallized from cold water to remove hydrochloric acid. This obtained crystal was dissolved in distilled water to a concentration of 0.08 per cent and 0.5 ml. of this solution were used in the experiments.

(*3*) *Method of Colorization*—To 0.5 ml. of the sample solution was added hydrochloric acid in various concentrations and the total volume was made up to 3.0 ml. To the mixture was added 0.5 ml. of 0.08 per cent diazobenzonium sulfonic acid and after 20 minutes the color was determined with a Hitachi photometer using filter, S 43, S 47, S 50 and S 53. As a control for measurement a mixture containing 3 ml. of hydrochloric acid of the same concentration as the sample solution and 0.5 ml. of 0.08 per cent diazobenzonium sulfonic acid was used.

The most suitable filter for each experiment is indicated in the remarks column in Table I. However, since the values obtained by filters other than S 47 did not show great differences from that of S 47, only the optical densities found with S 47 are given in Table I.

(*4*) *Paper Chromatography of the Urine of Rabbits Administered with Scatolylhydantoin*—Scatolylhydantoin was administered orally to rabbits in a dosage of 0.5 g. per kilogram of body weight and the urine was collected for 24 hours-period in a bottle, in which 5 ml. of concentrated hydrochloric acid was added before hand. The urine was concentrated in vacuo to about 10 ml. after being neutralized with 10 per cent NaOH, then chromatographed on Toyo Filter paper No. 51 with a mixture of acetic acid, butanol and water (1:4:5) using the ascending method.

(*5*) *Oxidation of Tryptophan and Scatolylhydantoin*—This experiment was carried out according to the procedure of Dalgliesh (5); 2.0 ml. (90 μM) of Tryptophan, or 2.0 ml. (40 μM) of scatolylhydantoin, 1.0 ml. (40 μM) of EDTA, 2.0 ml. (10 μM) of $FeSO_4$, 2.0 ml. (10 μM) of vitamin C, 0.8 ml. of 1 *M* phosphate buffer (pH 6.8) and 0.2 ml. of distilled water were mixed and the mixture was kept at 37° for two hours. After the incubation, ethanol was added in a final concentration of 60 per cent to remove the salts and evaporated in vacuo to a syrup. The syrup was dissolved in a small quantity of distilled water and used for the paperchromatography.

Results :

(*1*) *Relationship between Colorization and the Concentrations of Hydrochloric acid and the Samples*—The experiments were carried out at the various concentrations of hydrochloric acid and samples described, and the results are shown in Table I. It was found that the tone of the resulting color depends on the concentrations of both. 7-Hydroxyindole acetic acid gave a strong orange color immediately after addition of diazobenzonium sulfonic acid and the red tone increased by addition of hydrochloric acid. In the presence of higher concentrations of hydrochloric acid the color became more red-purple. In the cases of 5-hydroxyindole derivatives, however, a faint orange color appeared 10–20 minutes later and reached to maximum after 6 hours (Table II). The color given

TABLE I

The Comparison of Colorization for Various Compounds

(Numerial value show optical density with Filter S 47)

Concentration of HCl	0			0.6 *M*			1.0 *M*			2.0 *M*			4.0 *M*			
Sample(μ*M*)	1.0	0.5	0.25	1.0	0.5	0.25	1.0	0.5	0.25	1.0	0.5	0.25	1.0	0.5	0.25	
Indole acetic acid	Y 0.20	Y 0.15	Y 0.12	Y 0.10	Y 0.07	Y 0.05	Y 0.10	Y 0.07	Y 0.05	Y 0.10	Y 0.07	Y 0.05	Or 0.20	Y 0.12	Y 0.03	
7-Hydroxyindole acetic acid	Or 1.90	Or 1.70	Or 1.30				R-P 1.90	R-P* 1.00	R-P* 0.58	R-P* 1.90	R-P* 1.00	R-P* 0.62				*suitable filter S53
Serotonin	Or 0.18	Or 0.10	Or 0.05	P 0.07	P 0.02	–	P 0.04	P 0.02	±	P 0.04	P 0.03	±	P 0.04	P 0.03	±	
Indole	Y 0.13	Y 0.09	Y 0.07	R-P 0.20	R-P 0.14	R-P* 0.03	R-P* 0.18	R-P* 0.14	R-P* 0.09							*suitable filter S53
5-Methylindole	Y 0.18	Y 0.10	Y 0.05				Y-P 0.15	B-P 0.10	B-P 0.07	Br 0.28	B-P 0.20	B-P 0.08				
Scatole	Y 0.13	±	–	Y 0.07	Y 0.04	–				Or 0.03	Or 0.05	–	Or 0.10	Or 0.08	Or 0.04	
5-Hydroxyindole acetic acid	Or 0.13	Or 0.09	Or 0.05				P 0.03	P 0.02	–							
5-Hydroxytryptophan	Or 0.08	Or 0.05	Or ±				P ±	–	–							
Indole pyruvic acid	Y 0.05	Y 0.04	–				Or* 0.07	Or* 0.05	–				Or-P 0.10	Or-P 0.05		*suitable filter S43

Y: yellow, Or: orange, P: purple, R: red, B: blue, Br: Brown.

5-Methyltryptophan, indolecarbonic acid, indole lactic acid and abrin give no color.

7-Hydroxyindole acetic acid, 5-methylindole, scatole and indole give color immediately.

5-Hydroxyindole derivatives, indole acetic acid and indole pyruvic acid give color after ten minutes.

by these compounds is very weak comparing to that observed with 7-hydroxyindole acetic acid and moreover it was hardly detectable at a concentration of less than 0.25 μM. When the concentration of hydrochloric acid was raised to 1–4 *M*, serotonin, 5-hydroxyindole acetic acid and 5-hydroxytryptophan gave a purple color (Table I). However, the strength of the color occurring in the presence of diazobenzonium sulfonic acid alone was not decreased even by further addition of hydrochloric acid.

When the concentrations of the hydrochloric acid and the samples were raised, indole acetic acid, 5-methylindole and scatole gave a red acid diazoreaction after short time interval but the degree was less than one tenth of that found with 7-hydroxyindole acetic acid.

TABLE II

The Comparison of Strength of Color with Diazobenzonium Sulfonic Acid between Serotonin and 7-Hydroxyindole Acetic Acid

Time	0 *min.*	20 *mins.*	60 *mins.*	120 *mins.*	6 *hrs.*
7-OH-indole acetic acid (1 μM)		1.900	1.900	1.900	
Serotonin (1 μM)	0	0.140	0.240	0.310	0.590

(The strength of color is showed by the optical density with Filter S 47.)

(2) Experiments using Paperchromatography—(a) When 0.08 per cent diazobenzonium sulfonic acid was sprayed on the paper, 7-hydroxyindole acetic acid and 7-hydroxytryptophan gave reddish orange spots immediately, while 5-hydroxyindole derivatives gradually started to give reddish orange spots on drying. Indole acetic acid gave a yellow spot. (b) When Ehrlich's diazoreagent (containing 0.6 *M* HCl) was sprayed, 7-hydroxyindole derivatives and indole acetic acid gave red spots immediately, but 5-hydroxyindole derivatives gave red spots gradually on drying and the color was much weaker than those obtained with 7-hydroxyindole derivatives.

Therefore it is possible to identify 7-hydroxyindole derivatives by using both (a) and (b).

(3) Experiments on Scatolylhydantoin—As described above, paperchromatography was carried out with the urine of rabbits orally administered scatolylhydantoin. A red spot having an R_f of 0.63 appeared im-

mediately after 0.08 per cent diazobenzonium sulfonic acid or Ehrlich's diazoreagents was sprayed.

After oxidation of scatolylhydantoin according to the Dalgliesh's method, red spots corresponding to 7- and 5-hydroxyindole compounds were found with R_fs of 0.63 and 0.71 respectively.

II. Enzymatic Formation of 5- and 7-Hydroxyindole Compounds from Scatolylhydantoin

Materials and Methods:

(*1*) *Enzyme Preparation*—After the rabbits were slaughtered by bleeding, the livers were isolated, and ground with an equal amount of quartz sand, followed by the extraction with an equal quantity of 30 per cent sucrose at 0° for one hour. The insoluble residue was removed by centrifugation at 9,800 × *g*. for 20 minutes, and the supernatant was used as the enzyme preparation.

(*2*) *Reaction Mixture*—The following conditions were used unless otherwise specified:

1 *M* Phosphate buffer (pH 8.0)	0.3 ml.
TPN (20 μg./0.1 ml.)	0.1 ml.
Citrate (1 mg./0.2 ml.)	0.2 ml.
Scatolylhydantoin (2 μM/ml.)	1.0 ml.
Enzyme preparation	1.0 ml.

The total volume was 3.0 ml., and the reactions were carried out at 38° for 15 minutes.

Brown test tubes were used for incubation, to avoid the effect of ultra-violet ray on the hydroxyindole derivatives.

Reactions were stopped by the addition of 0.5 ml. of 50 per cent trichloroacetic acid to the reaction mixture and filtered. 2 ml. of Ehrlich's diazoreagent was added to 2 ml. of the protein-free filtrate and the acid diazoreaction was carried out. After 20 minutes the optical density was measured with a Hitachi photocolorimeter using filter S 53 and the enzyme activity was compared. Under these conditions the presence of hydrochloric acid strongly inhibited color formation with 5-hydroxyindole derivatives indicating that its effect on optical density was negligible. Accordingly the observed optical density could be attributed to 7-hydroxyindole derivatives alone.

Results:

(*1*) *Participation of TPNH⁺ in the Hydroxylation Reaction*—The participation of $TPNH^+$ in the hydroxylation of aromatic compounds was first confirmed by Udenfriend (*6*). As shown in Table III, $TPNH^+$ also participated in the enzymatic hydroxylation of scatolylhydantoin but $DPNH^+$ was found to be without effect.

(*2*) *Studies on the Properties of Enzyme*—The optimal condition for the maximum activity was obtained from the following figures.

(*3*) *Studies on the Inhibitor*—As shown in Table IV the enzyme activity was inhibited by the presence of the various metal catchers but inhibitors of the SH group were found to have no effect.

TABLE III

Participation of $TPNH^+$ *in the Hydroxylation of Scatolylhydantoin*

Addition	log I/I_0
None	0.020
Citrate (1 mg.)	0.130
TPN (20 μg.)	0.050
DPN (400 μg.)	0.050
TPN (20 μg)+Citrate (1 mg.)	0.340
DPN (400 μg)+Citrate (1 mg.)	0.120
DPN (400 μ)+Malate (1 mg.)	0.080

The reaction was carried out for 30 minutes, unless specified.

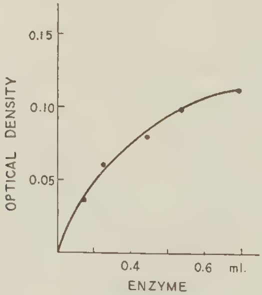

FIG. 1. Quantity of enzyme.

Except for the quantity of enzyme, this experiment was carried out as described above.

III. *Hydroxylation of Indole Compounds in Liver Extract System*

The hydroxylation of various indole compounds was carried out on a large scale using 20 times the quantity described above, but the *M*/10 phosphate buffer extract (pH 7.2) was used as enzyme. After 2 hours of incubation the reaction mixture was deproteinized by the addition of

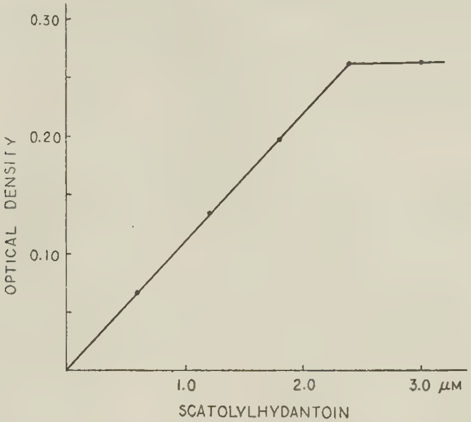

FIG. 2. Quantity of substrate.
Except for the quantity of substrate, carried out as described above.

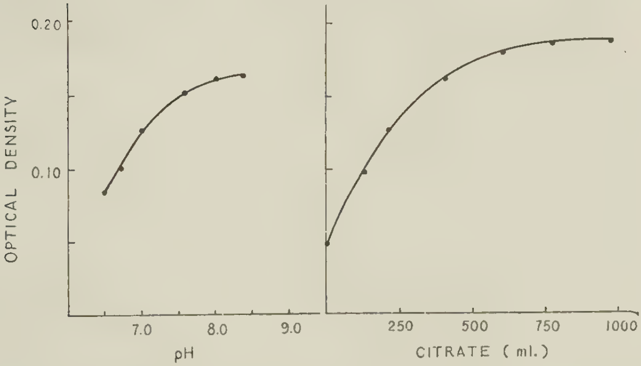

FIG. 3. pH curve.
Except for the pH of reaction, carried out same condition described above.

FIG. 4. Quantity of citrate.
Except for the quantity of citrate, carried out as described above.

ethanol, and then evaporated *in vacuo* at room temperature in a dark room.

The system for scatolylhydantoin was slightly different. This

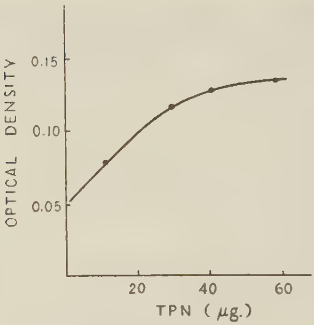

FIG. 5. Quantity of TPN. Except for the quantity of TPN, carried out as described above.

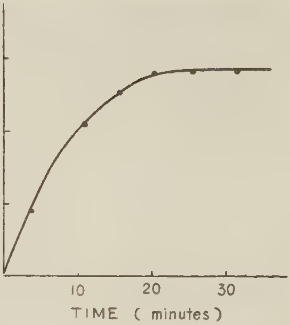

Fig. 6. Incubation time Except for the incubation time, carried out as described above.

TABLE IV

Effects of Various Inhibitors on Enzyme Activity

Inhibitor	Final concentration *M*	Inhibition %
Nitroso-R-salt	10^{-3}	93
KCN	〃	30
α-*α*′ Dipyridyl	〃	78
o-Phenanthroline	〃	78
NH_2OH	〃	51
EDTA	〃	35
Azide	〃	52
PCMB	10^{-4}	17
$HgCl_2$	2×10^{-3}	0
Monoiodoaceticacid	10^{-3}	0
Cysteine	〃	0
GSH	〃	0

compound was incubated for 15 minutes, and after deproteinization the supernatant was extracted with 1.5 volume of ethyl acetate. Distilled water was added until the ethyl acetate layer was separated clearly, which was then evaporated *in vacuo*. The concentrated fluid was spotted on a paper in the darkroom and developed with a mixture of acetic acid, butanol and water (1 :4 :1). The results are shown in Table V.

Table V

Hydroxylation of Various Indole Compounds by Liver Extract

Substrate	R_f		Notice
	7-Hydroxy compound	5-Hydroxy compound	
Scatolylhydantoin	0.62	0.82	Scatylylhydantoin, tryptamine, indolepropionic acid, indolelactic acid and tryptophan were hydroxylated in 5 and 7 position of indole nucleous by Dalgliesh's method.
Tryptamine	0.47	0.64	
Indolepropionic acid	0.75	0.72	
Indolelactic acid	0.56	not found	
Tryptophan	not hydroxylated	not found	
Serotonin	not hydroxylated		

Indolepyruvic acid, indoleacrylic acid, 5-methylindole, 5-methylindole-2-carboxylic acid, isatin and N-α-methyltryptophan were not hydroxylated.

DISCUSSION

Indole acetic acid, 5- and 7-hydroxyindole derivatives were chromatographically shown by Stowe (*2*) to give the acid diazoreaction. From results described above it was confirmed that 7-hydroxyindole derivatives and indole acetic acid give the positive acid diazoreaction on the wet paper after spraying with Ehrlich's diazoreagents while the 5-hydroxyindole derivatives only become apparent gradually on drying. Therefore if the colouring time is disregarded, the above substances cannot be distinguished from others. When diazobenzonium sulfonic acid alone is used, indole acetic acid gives a yellow spot while 7-hydroxyindole derivatives give the orange red spots on the wet paper and 5-hydroxyindole derivatives give spots only gradually on drying.

When the acid diazoreaction is carried out in the test tube, it is preferable to use diazobenzonium sulfonic acid. In this case the 7-hydroxyindole derivatives give an orange color earlier and greater strength than 5-hydroxyindole derivatives, while indole acetic acid give a yellow color.

7-Hydroxyindole derivative was found paperchromatographically in the urine of scatolylhydantoin administered rabbits. However, when scatolylhydantoin was incubated with liver extract in the presence of TPN and citrate, 5- and 7-hydroxyindole derivatives could be detected.

In addition, corresponding hydroxylated compounds at the 5- and 7-positions were demonstrated to be formed from indole lactic acid, indole propionic acid and indole ethylamine by liver extract in the presence of TPN and citrate.

In the previous paper it was reported that 7-hydroxyindole derivative was present in the extract of bufo skin. However this observation was shown to be incorrect by the present experiment, which indicated that the acid diazoreaction given by the bufo skin extract appeared only after the paper dried. Therefore this spot must be attributed to the presence of 5-hydroxyindole derivative.

SUMMARY

1. The relationship between an acid diazoreaction and the concentration of each reagents was investigated and the cause of the discrepancy between the data obtained by Stowe and Ichihara *et al.* was clarified.

2. In the acid diazoreaction, 7-hydroxyindole derivatives can be easily distinguished from 5-hydroxyindole derivatives and indole acetic acid by using both the diazobenzonium sulfonic acid and Ehrlich's diazoreagents.

3. Indole propionic acid, indole lactic acid, indole ethylamine and scatolylhydantoin were converted to their hydroxylated compounds at the 5 or 7 position by liver extract.

4. The enzyme responsible for the hydroxylation of the indole derivative was studied. $TPNH^+$ was found to participate in this reaction but $DPNH^+$ did not. Inhibitors in this reaction were also investigated.

5. The description of 7-hydroxyindole derivative in bufo skin extract in the previous paper must be corrected.

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The Journal of Biochemistry, Vol. 44, No. 10, 1957

THE EFFECT OF VARIOUS ANTIBACTERIAL SUBSTANCES ON GLUCOSE RESPIRATION OF MICROCOCCUS PYOGENES VAR. AUREUS WITH SPECIAL REFERENCE TO THEIR GROWTH INHIBITORY ACTION

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In a previous study of the inhibition of growth by antibacterial substances the mode of action of these agents was analysed both qualitatively and quantitatively (*1*). In the present experiment the inhibitory effects of some of these antibacterial agents on the respiration of glucose by *Micrococcus pyogenes var. aureus* were investigated and their bactericidal activities under identical conditions were also determined. On comparing the results of these experiments with those of previous studies of bacteriostasis, some information on the interrelationship among respiration inhibition and bacteriostatic and bactericidal actions was obtained.

METHODS AND MATERIALS

The antibacterial substances used in this experiment were selected from the substances used in the previous work for their typical antibacterial characteristics. These substances include alcohol, phenols, mercurials, sulfanilamides, dyes, antibiotics and other agents; all of which are shown in Fig. 3.

The organism used was *Micrococcus pyogenes var. aureus Terashima* cultured overnight on nutrient agar (pH 7.4) at 37°. Bacteria were harvested, washed 5 times with *M*/15 phosphate buffer at pH 7.4, and suspended in regenerated buffer. The amount of cells thus obtained was from 5 to 8 mg. dry weight per ml.

The rate of oxygen uptake was measured by the Warburg manometer, with 0.5 ml. of 10 per cent potassium hydroxide in the center well, 0.5 ml. of *M*/2 glucose in the side arm and 2 ml. of cell suspension in the main chamber; gas phase being filled with air. Prior to the manometric measurement, 0.5 ml. of the solution of test substance was added into the main chamber and shaked for 15 minutes at 37°. Immediately after the addition of glucose from the side arm the oxygen uptake was measured for about 1 hour at intervals of 5 minutes.

Bactericidal activity of a given substance was determined as follows: in a test tube there was added 2 ml. of cell suspension and 1 ml. of test solution. The number of viable cells was measured by the capillary tube method of Yanagita (*2*) before and after 2 hour incubation at 37°. The concentration killing half of the population was designated as the criterion of bactericidal activity of the test substances. It was determined by using various concentrations of the test substances.

RESULTS

Inhibition of Glucose Respiration—The effect of varied concentrations of $HgCl_2$, for example, on the respiration of the bacteria is shown in Fig. 1.* This type of inhibition was observed with all of the other substances,

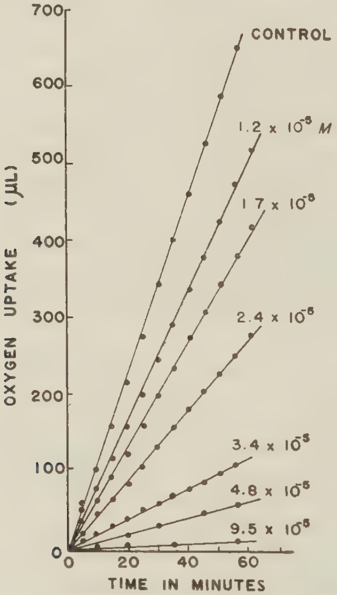

FIG. 1. Oxygen uptake in the presence of varied concentration of sublimate.

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* Since the endogenous respiration was less than 5 per cent of the substrate respiration, the rate of the oxygen-uptake including the endogenous respiration was taken in the following quantitative analysis of the respiration inhibition caused by a given substance. The Q_{O2} of the control substrate respiration was approximately 60 cmm./min./mg. dry weight.

except 2,4-dinitrophenol and salicylic acid which exhibited an accelerating effect within a certain limit of concentration. Acceleration of respiration caused by 2,4-dinitrophenol, as was already discussed by Clifton (*3*), was observed at concentrations between 5×10^{-4} and 8×10^{-3} molar, the degree of acceleration being 25 per cent at its maximum. A similar result was obtained with salicylic acid at concentrations between 3×10^{-3} and 3×10^{-2} molar.

The slope of the oxygen uptake curve at respective concentration of the substance in Fig. 1 was taken as the rate of respiration, and the degree of inhibition, H, was defined as follows:

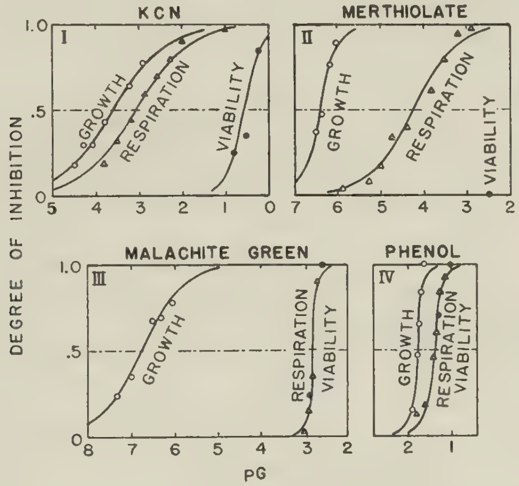

FIG. 2. H-pG-curves for growth inhibition, respiration inhibition and bactericidal action.

I: Potassium cyanide, II: merthiolate, III: malachite green, and IV: phenol.

$$H=1-\frac{V_G}{V},$$

where V and V_G are the rate of respiration in the absence and presence of the substance respectively. To illustrate the functional relationship between H and the concentration of the added substance we have plotted the former on the ordinate and the latter (in terms of moles per liter) on the abscissa in a negative logarithmic scale as shown in Fig. 2. The curves thus obtained, which we call "H-pG-curve," were found in all cases to represent sigmoid curves. The similarity of the curves as compared with those obtained in growth inhibition experiments is clearly illustrated in the figures. The relationship between H and the concentration, G, of the added substance can quite generally be expressed by the following equation given by Tamiya *et al.* (*1*) for growth inhibition experiments:

$$H=\frac{G^n}{\phi^n+G^n},$$

where n and ϕ are constants; the former value determines the shape (steepness) and the latter the lateral position (representing concentration causing 50 per cent inhibition) of the H-pG-curve.

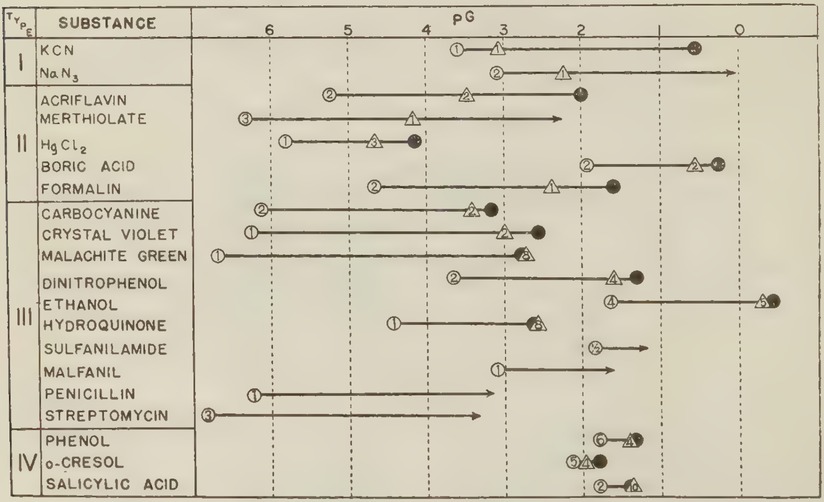

FIG. 3. Comparison of pG-values for growth inhibition (○), respiration inhibition (△) and bactericidal action (●) of various antibacterial substances.

Numbers shown in circular and triangular marks represent n-values for each activity of the test substances. The concentrations of these substances are expressed in terms of moles per liter, except those of penicillin and streptomycin. The latters are given in terms of g. per ml.

The n- and ϕ-values obtained for each substance are shown in Fig. 3, in which the position of the triangular mark represents pϕ-value (ϕ-

value expressed in terms of negative logarithm) and the number appearing in the mark represents n-value. Those values for growth inhibition obtained by one of the authors (*4*) are also shown in the figure by a circular mark. It can be seen from these experiments that the n-values of various substances obtained for respiration and growth inhibition are in fairly good agreement, with some exceptions, and that $p\phi$-values for respiration inhibition are more or less lower than those for growth inhibition ; this fact will be discussed later in detail.

Bactericidal Action—The bactericidal activities at varied concentrations of $HgCl_2$, for example, are tabulated in Table I. Percentage activities plotted against concentration of some of the substances tested are shown in Fig. 2. With such a representation the concentration which cause 50 per cent reduction of viable cells was obtained for each substance. The position of the black circles appearing in Fig. 3 stands for this concentration of test substances and the arrows indicate that no significant bactericidal activity was exhibited with the concentrations tested.

TABLE I

Bactericidal Action of Sublimate

Concentration	Number of colonies per ml.	Percentage activity
Control	39×10^8	
1.6×10^{-5} *M*	41×10^8	0
3.1×10^{-5}	40×10^8	0
6.2×10^{-5}	26×10^8	33
1.3×10^{-4}	1×10^8	97
2.5×10^{-4}	0.1×10^8	100
5.0×10^{-4}	$<0.1\times10^8$	100

As was expected, the $p\phi$-value for bactericidal activity obtained for each substance is lower than the values for growth and respiration inhibition. A detailed discussion on the comparison for these three values is presented in the next paragraph.

DISCUSSION

Although the cell density and the composition of the medium is quite different in growth and respiration inhibitions and bactericidal action, a comparison of these activities for each antibacterial substance tested may be of some significance in determining the participation of respiration inhibition in the mode of action of these substances. By comparing the pϕ-values for bacteriostasis, respiration inhibition and bactericidal action, as shown in Fig. 3, substances were found to fall into four groups:

I. Substances having close high activities for growth and respiration inhibition but not for bactericidal action. Of the substances tested cyanide and azide, known as specific inhibitors of respiration, fall into this group. Fig. 2–I shows the various actions of cyanide expressed in terms of H-pG-relationships. Both the values n and pϕ causing inhibition of growth and respiration are very similar despite the about 10,000 fold difference in cell density, while the bactericidal activity is far weaker. The fact suggest that the inhibition of respiration by these substances is primarily concerned with the inhibition of growth, and that a far higher concentration is apparently necessary to bring about bactericidal action. Since the viable count to determine the said activity is measured in drug-free medium, it seems likely to cause a reversible release from the toxic action of the substance.

II. Substances having widely different activities for growth, respiration and viability. Two substances, merthiolate and acriflavin, were found in this group. Merthiolate is the most straight foreward example and its H-pG-curves are shown in Fig. 2–II. With this group of substances the inhibition of respiration seems not to play a primary role in the mechanism of growth inhibition and even the concentration causing complete inhibition of respiration does not bring about the total death of the bacterial cell. Sublimate ($HgCl_2$), boric acid and formalin could be classified into this group, but they are the substances which are considered to be rather an intermediate group between groups II and III.

III. Substances having a much higher activity for growth inhibition. In this group of substance hydroquinone, dinitrophenol, ehtanol, crystal violet, malachite green and carbocyanine are found. Characteristic of these substances is the appearance of close pϕ-values for respiration inhibition and bactericidal action, though at a very low value; typical example of H-pG-curves for malachite green are presented in Fig. 2–III. Sulfanilamide, malfanil, penicillin and streptomycin may also be regarded as special cases of the same type. No significant activity for respiration inhibition and bactericidal action are observed with these substance in spite of their remarkable effect towards bacterial growth. It can be concluded from these observations that with this group of substances the inhibition of growth is primarily dependent upon the inhibition of some process(es) other than glucose respiration inhibition.

IV. Substances with relatively close coincidence of these three activities. Phenol, o-cresol, and salicylic acid can be classified into this group (Fig. 3). A close bundle of H-pG-curves obtained for one of these substances are shown in Fig. 2–IV. In this case it is suggested that these substances bring about abrupt and irreversible action on the bacterial cell.

SUMMARY

The inhibitory activities of various antibacterial substances on the respiration, growth and viability of *M. pyogenes* were compared on the basis of activity-concentration relationships. From this comparison, it was revealed that antibacterial substances could be classified into four groups in this respect. These groups are discussed from the point of view of mode of action of the substances tested.

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The Journal of Biochemistry, Vol. 44, No. 10, 1957

STUDIES ON THE SYNTHESIS OF HIPPURYLANILIDE BY PLANT ENZYME

I. EFFECT OF METAL SALTS ON THE ANILIDE SYNTHESIS BY PAPAIN

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Since the discovery of the enzymatic synthesis of acylamino acid anilide (*1*), much attention has been given to the optical specificity of the enzyme and to the various factors influencing the synthetic reaction (*2–7*). However, there has been no report as yet which deals with the effect of metal salts on the synthesis. The present author has tested the effect of various metal salts on the papain-catalyzed synthesis of hippurylanilide both in the presence and in the absence of activators such as cysteine, potassium cyanide, and rongalit C.

The effect of metal salts on the papain-catalyzed hydrolysis of gelatin was first described by Krebs (*8*) in 1930. He reported that copper, cadmium, and mercury salts inhibited the hydrolysis, while manganese, cobalt, and aluminum had no effect. Later, stimulatory effect of ferrous and ferric ions on the hydrolysis of gelatin by papain was reported by Maschmann (*9, 10*). Almost similar results have been obtained also in the present experiments for hippurylanilide synthesis by papain. Thus, in the absence of the activators, cupric, mercuric, cadmium, or stannic ion inhibited the synthetic reaction, while the addition of stannous, ferrous, or ferric ion was strongly stimulatory. In the presence of activator, the yield of the anilide was increased to 3- to 4-fold, and most heavy-metal ions were still inhibitory on the synthetic reaction, whereas stimulatory effect of stannous, ferrous, or ferric ion was no longer observed.

EXPERIMENTAL

Enzyme Solution—A suspension of 3.6 g. of papain* in 12 ml. of water was centrifuged at 4,000 r.p.m. for 10 minutes and the supernatant was used as enzyme. In some instances the dialyzed preparation was employed which was obtained by suspending 7.2 g. of papain in 6 ml. of water and dialyzing against 6 liter of de-ionized water for 24 hours. After dialysis the enzyme solution was made up to the volume of 12 ml. with water and centrifuged at 4,000 r.p.m., and the supernatant was used as enzyme.

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* Papain (1:350) " E. Merck, " Darmstadt:

Substrate Solution—In 200 ml. of water was dissolved 30 g. of hippuric acid together with 49 g. of sodium citrate, the pH of the solution being 4.8.

Aniline Solution—To 3.8 ml. of aniline were added 15 ml. of 2 *N* HCl and water to make a total volume of 20 ml.

Metal Salt Solution—The salts tested were $CuSO_4$, $Cd(C_2H_3O_2)_2$, $Hg(C_2H_3O_2)_2$, $SnCl_2$, $SnCl_4$, $MnCl_2$, $FeSO_4$, $Fe_2(SO_4)_3$, $CoCl_2$, $MgSO_4$, and $AlCl_3$.

Enzymatic Synthesis of Hyppurylanilide—A mixture of 1.0 ml. of enzyme, 2.0 ml. of substrate, 2.0 ml. of aniline solution, 1.0 ml. of 1 *M* citrate buffer of pH 4.8, 0.5 ml. of 1 *N* NaOH, and water to be a total volume of 10 ml. was incubated at 37° for 24 hours. The solution of metal salts to be tested was added in place of water to the mixture so as the final concentration of the salts was *M*/100, *M*/1000, or *M*/10000. In the experiments with activated enzyme, the incubation mixture contained *M*/200 cysteine, potassium cyanide, or rongalit C. The anilide formed was determined gravimetrically (*4*, *7*).

RESULTS

In the absence of activator, papain catalyzed the synthesis of a hippurylanilide, *i.e.*, 3–7 per cent of the theoretical value, when ordinary distilled water was used as solvent. The yield of the anilide was increased to 18 per cent of the theoretical by the use of de-ionized water.* All the following experiments were performed with de-ionized water. The results obtained are summarized in Table I.

Effect of Metal Salts in the Absence of Activator

Non-dialyzed Enzyme—As shown in Table I, Cu^{++}, Cd^{++}, Hg^{++}, and Sn^{++++} exhibited notable inhibition of the anilide synthesis. The addition of Cu^{++} caused 70 per cent inhibition at a final concentration of 10^{-2}–10^{-3} *M* and 60 per cent at 10^{-4} *M*. Cd^{++} was markedly inhibitory at 10^{-2} *M*, moderately at 10^{-3} *M*, and had almost no effect at 10^{-4} *M*. Hg^{++} was so inhibitory that it caused 65 per cent inhibition at 10^{-2} *M* and 30 per cent at 10^{-3} *M*. Mn^{++} and Co^{++} were only less inhibitory. Contrary to these ions, Fe^{++}, Fe^{+++}, and Sn^{++} showed remarkably stimulatory

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* Mono-bed type apparatus of Organo Co. Ltd., Tokyo: Amberites IR-120 and IRA-410 were used as ion exchanger.

TABLE I

Effect of Metal Ions on the Papain-catalyzed Synthesis of Hippurylanilide

The yield is given in per cent of the theoretical.

Metal ion final concentration 10^{-n} M		Yield of hippurylanilide									
Activator→		None (control)				Cysteine		KCN		Rongalit C	
Papain→		Non-dialyzed		Dialyzed		Non-dialyzed		Non-dialyzed		Non-dialyzed	
Yield↘		%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio
None (control)		18.1	100	15.1	100	69.7	100	52.5	100	56.0	100
Cu^{++}	2	5.6	32	2.6	17	11.0	15	9.2	17	8.5	15
	3	5.2	28	5.6	37	14.0	20	6.6	12	11.0	19
	4	7.0	39	11.8	78	52.0	74	22.8	43	61.0	109
Cd^{++}	2	7.3	40	6.8	45	35.6	51	25.2	48	23.7	42
	3	11.6	64	14.5	96	66.8	96	38.0	72	54.7	96
	4	17.2	95	—	—	78.0	112	46.0	87	64.0	114
Hg^{++}	2	6.3	35	1.3	8	6.7	9	7.5	14	8.4	15
	3	13.0	72	5.4	36	67.0	96	8.0	15	59.5	106
	4	11.0	61	17.5	116	86.0	123	29.5	56	67.0	120
Sn^{++}	2	64.5	365	53.0	350	80.0	115	58.5	111	50.5	90
	3	29.5	166	17.0	112	75.5	108	53.5	102	58.5	104
	4	20.0	110	20.3	132	71.0	101	—	—	—	—
Sn^{++++}	2	10.0	55	12.3	81	57.5	82	34.2	65	48.0	85
	3	12.7	67	14.2	94	—	—	—	—	—	—
	4	13.2	73	17.5	116	—	—	—	—	—	—
Mn^{++}	2	16.6	91	11.7	77	74.0	106	41.0	78	57.5	102
	3	16.1	89	13.9	92	—	—	46.0	87	62.0	110
	4	14.5	80	—	—	—	—	49.0	93	67.0	120
Fe^{++}	2	54.2	300	36.0	240	69.0	99	56.0	106	52.5	94
	3	36.6	201	24.8	160	—	—	—	—	—	—
	4	30.0	166	17.5	116	—	—	—	—	—	—
Fe^{+++}	2	47.0	260	20.1	133	62.5	89	54.7	104	57.0	102
	3	32.3	178	20.1	133	—	—	—	—	—	—
	4	22.0	122	20.0	132	—	—	—	—	—	—
Co^{++}	2	14.7	81	9.2	61	77.2	110	40.0	76	56.5	101
	3	16.0	88	12.4	82	—	—	51.5	97	55.0	98
	4	17.0	94	16.0	106	—	—	51.5	97	61.4	110
Mg^{++}	2	22.6	121	16.3	108	76.6	109	55.0	105	59.0	105
	3	22.2	120	14.2	94	—	—	—	—	—	—
	4	18.0	100	—	—	—	—	—	—	—	—
Al^{+++}	2	27.0	149	15.1	100	74.5	107	46.7	89	53.5	95
	3	23.6	130	17.2	114	—	—	—	—	—	—
	4	18.8	104	—	—	—	—	—	—	—	—

effect on the anilide synthesis, but Al^{+++} and Mg^{++} a weak stimulation. The yield of the anilide increased about 3 times in the presence of Fe^{++} at 10^{-2} *M*, 2 times at 10^{-3} *M*, and 1.6 times at 10^{-4} *M*. Fe^{+++} was also stimulatory so that it caused increase in the yield of the anilide 2.6 times at 10^{-2} *M*, 1.8 times at 10^{-3} *M*, and 1.2 times at 10^{-4} *M*.

The highest yield of the anilide was obtained in the presence of Sn^{++} at 10^{-2} *M*, which at 10^{-3} *M*, however, caused only 1.6 times increase of the yield and at 10^{-4} *M* almost no effect.

The stimulation by Al^{+++} was so weak that at 10^{-2} *M* it caused only 1.5 times yield, at 10^{-3} *M* 1.3 times, and at 10^{-4} *M* no effect. Mg^{++} exhibited a weak stimulation at 10^{-2}–10^{-3} *M*.

Dialyzed Enzyme—After 24 hours dialysis, the activity of the enzyme was reduced to such an extent that it produced only 15 per cent yield of the anilide, despite the fact that the dialyzed preparation contained about twice as large amount of protein as did the non-dialyzed. Cu^{++}, Cd^{++}, and Hg^{++} exhibited a strong inhibition on the anilide synthesis by the dialyzed enzyme, the degree of inhibition being higher for the dialyzed than for the non-dialyzed. The similar tendency was observed also in the case of the inhibition by Mn^{++} or by Co^{++}. On the contrary, the inhibitory effect of Sn^{++++} was rather weaker for the dialyzed enzyme than for the non-dialyzed.

Fe^{++} and Fe^{+++} had also a stimulatory effect, but the degree of stimulation was lower for the dialyzed enzyme than for the non-dialyzed. Thus, Fe^{++} caused 2.4 times increase in the yield of the anilide at 10^{-2} *M*, but it had no effect at 10^{-4} *M*. Fe^{+++} caused 1.3 times increase at 10^{-2}–10^{-4} *M*.

The stimulation by Sn^{++} was noted to the same extent both for the dialyzed and non-dialyzed enzyme: it was 3.5 times increase at 10^{-2} *M*.

Al^{+++} and Mg^{++} had almost no effect on the action of the dialyzed enzyme.

Effect of Metal Salts in the Presence of Activator

The test was made with non-dialyzed enzyme preparation which was activated by the addition of cysteine, KCN, or rongalit C. As shown in Table I, cysteine-activated papain catalyzed the synthesis of about 4 times the amount of hippurylanilide as compared with the action of papain in the absence of activator. About 3 times increase in the yield of the anilide was observed also with KCN or rongalit C as activator.

Cysteine-activated Enzyme—The yield of the anilide in the presence of 10^{-2}–10^{-3} *M* Cu^{++} was less than that for the non-activated enzyme, *i.e.*, at this level of concentration Cu^{++} still exhibited a strong inhibition and the addition of cysteine could not restore the enzymatic activity. At 10^{-4} *M* Cu^{++}, however, cysteine showed a notable reactivation of the enzyme. In the presence of cysteine, Cd^{++} was inhibitory at 10^{-2} *M* and almost without effect at 10^{-3}–10^{-4} *M*. Hg^{++} was inhibitory at 10^{-2} *M* to the same extent as Cu^{++}, but at 10^{-3}–10^{-4} *M* it seemed to have no or rather a slight stimulatory effect on the anilide synthesis.

KCN-activated Enzyme—Contrary to the results obtained with cysteine-activated enzyme, a strong inhibition was always noted with Cu^{++}, Hg^{++}, Cd^{++}, and Sn^{++++} even at 10^{-3}–10^{-4} *M*. Thus, KCN appears to be less capable of reactivating enzyme that has been inhibited by heavy-metal salts.

Rongalit C-activated Enzyme—The addition of Cu^{++}, Cd^{++}, and Hg^{++} showed similar inhibitory effects as those observed with cysteine-activated enzyme.

It should be noted here that the addition of Fe^{++}, Fe^{+++}, Sn^{++}, and Al^{+++}, all of which showed a stimulatory effect on the action of the enzyme in the absence of activator, was almost without effect on the enzyme activated by cysteine, KCN, or rongalit C.

DISCUSSION

Krebs (*8*) studied the effect of metal salts on the hydrolysis of gelatin as catalyzed by papain, and it was reported that Cu^{++}, Cd^{++}, and Hg^{++} were inhibitory while Fe^{++}, Al^{+++}, Co^{++}, and Mn^{++} had no effect. The present investigation dealing with the papain-catalyzed synthesis of hippurylanilide has revealed, as shown in Table I, that Cu^{++}, Cd^{++}, and Hg^{++} are inhibitory also on the synthetic reaction, while Fe^{++}, Fe^{+++}, and Sn^{++} have strong accerelating effects on the synthesis, and Co^{++} and Mn^{++} appeared to be somewhat inhibitory.

The inhibitory effect of metal ions such as Cu^{++}, Cd^{++}, Hg^{++}, and Sn^{++++} on the papain-catalyzed synthesis of hippurylanilide may be interpreted in terms of combination of these ions with sulfhydryl group of papain, causing inactivation of the enzyme. In the presence of an activator such as cysteine, the metal ions are trapped by the activator so that their inhibitory effect on the enzymatic action should be weakened. The only limited reactivation by KCN may be due to a weak combining potency of KCN with the metal ions.

The stimulatory effect of ferrous ion on the papain-catalyzed hydrolysis of gelatin was first reported by Maschmann (*9*). In a subsequent communication (*10*) he showed that ferric ion had also stimulatory effect. The results of the present experiments concerning the stimulatory effects of ferrous and ferric ions on the hippurylanilide synthesis are consistent with those obtained by Maschmann for gelatin hydrolysis. For the explanation of the observed stimulation by ferrous ion, Maschmann proposed a hypothetical mechanism that ferrous ion combines with some inactivating substance, "*Begleitstoff*," which he postulated to be contained in the crude papain preparation. He demonstrated that when the purified enzyme was used ferrous ion exerted little or no stimulatory effect on gelatin hydrolysis. His interpretation was that the purification of the enzyme caused removal of the "*Begleitstoff*" from the preparation. The fact that stimulation of hippurylanilide synthesis by ferrous ion is less noticeable with the dialyzed enzyme than with the original preparation (Table I) seems to support Maschmann's mechanism. Nevertheless, further investigation will be needed for the elucidation of the mechanism, in view of the recent reports on the Fe-activation of several enzyme systems (*11–13*).

SUMMARY

1. The enzymatic synthesis of hippurylanilide by papain is greatly stimulated, even in the absence of activator, by the use of de-ionized water as solvent.

2. In the absence of activator, the synthetic reaction by papain solution, dialyzed or not dialyzed, is markedly inhibited by the addition of metal ions such as Cu^{++}, Cd^{++}, Hg^{++}, and Sn^{++++}, and it is notably stimulated by Sn^{++}, Fe^{++}, and Fe^{+++}. The stimulatory influence is lowered in the dialyzed enzyme. The addition of Al^{+++} or Mg^{++} has also somewhat stimulatory tendency, which disappears in the dialyzed papain solution.

3. When cystein, potassium cyanide, or rongalit C is added to the enzyme preparation, the yield of hippurylanilide is increased 3–4 times as compared with that observed in the absence of the activator. The synthetic reaction in the presence of the activator is also inhibited by Cu^{++}, Cd^{++}, and Hg^{++}. In the presence of the activator Fe^{++}, Fe^{+++}, or Sn^{++} has no stimulatory effect on the anilide synthesis.

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STUDIES ON THE SYNTHESIS OF HIPPURYLANILIDE BY PLANT ENZYME

II. EFFECT OF METAL SALTS ON THE ANILIDE SYNTHESIS BY FICIN

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In a previous communication (*1*), it has been reported that various metal ions show stimulatory or inhibitory effect on the hippurylanilide synthesis catalyzed by papain. The synthesis of acylamino acid anilides by ficin was first reported by Utzino and Yoneya (*2–4*), and subsequently studied by Nakao (*5*) and others (*6–8*). The present paper deals with the effect of various metal ions on the ficin-catalyzed synthesis of hippurylanilide in the presence or in the absence of activators such as cysteine, potassium cyanide, and ronglit C. These activators had been shown to have stimulatory effect on the anilide synthesis catalyzed by papain, but it has been found that they are almost without effect on the action of ficin. Dialysis has resulted in no appreciable change in the synthetic activity of ficin. A number of metal ions exhibited an inbititory effect on the synthesis of hippurylanilide by ficin, while none of the metal salts tested showed stimulation.

EXPERIMENTAL

Acetone powder of fig sap was prepared by the method of Yoneya (3). An amount of 1.8 g. of ficin acetone powder was dissolved in 12 ml. of water and used as the enzyme solution. Sometimes the dialyzed preparation was employed, which was prepared in the following manner. A solution of 1.8 g. of ficin acetone powder in 5 ml. of water was dialyzed against 6 liter of de-ionized water for 24 hours. After dialysis the enzyme solution was made up to a volume of 12 ml. with de-ionized water. The composition of the test mixture and the experimental procedure for the synthesis and determination of hippurylanilide were entirely the same as those described in the experiment with papain (*1*). The de-ionized water (*1*) was used also for the present experiment.

RESULTS AND DISCUSSION

As shown in Table I, the synthetic activity of ficin was hardly reduced by dialysis.

The addition of cysteine, potassium cyanide and rongalit C as activator had almost no effect. It follows that the solution of ficin used for the present experiment was already in its full active state before the activating treatment.

Effect of Metal Salts in the Absence of Activator

As shown in Table I, Cu^{++}, Cd^{++}, Hg^{++}, and Mn^{++} exhibited inhibition of hippurylanilide synthesis. The degree of inhibition of ficin by Cu^{++} was almost the same as that of papain. Thus, Cu^{++} caused more than 90 per cent inhibition at a final concentration of 10^{-2} *M*, 76 per cent at 10^{-3} *M* and 50 per cent at 10^{-4} *M*. Cd^{++} inhibited the synthesis to an extent of 55 per cent at its final concentration of 10^{-2} *M*, but it exhibited no inhibitory effect at lower concentrations of 10^{-3}–10^{-4} *M*, a result different from that for papain. The inhibition by 10^{-2}–10^{-3} *M* Hg^{++} was 95 per cent, being stronger than that for papain, while 10^{-4} *M* Hg^{++} caused 50 per cent inhibition as it was the case for papain. Mn^{++} was weakly inhibitory. It should be noted here that Fe^{++}, Fe^{+++}, Sn^{++}, Mg^{++}, and Al^{+++}, all of which had exhibited stimulatory effect on the synthetic action of papain, had no appreciable effect on ficin. The addition of Co^{++} had also no effect.

Effect of Metal Salts in the Presence of Activator

Dialyzed enzyme preparation was employed in this case. The addition of Cu^{++}, Cd^{++}, and Hg^{++} exhibited inhibition of the anilide synthesis by ficin even in the presence of activator, as already observed with papain.

Cysteine-activated Enzyme—Cu^{++} inhibited synthetic action of the activated ficin in a manner almost similar to that for the non-activated enzyme. The addition of Cd^{++} exhibited inhibitory effect at 10^{-2} *M* only, no reactivation by cysteine being noted. However, at a lower concentration of 10^{-3} *M* the activity was recovered by cysteine.

At a final concentration of 10^{-2}–10^{-3} *M* the inhibition by Hg^{++} was less noticeable for the cysteine-activated ficin than for the non-activated, and at 10^{-4} *M* no inhibitory effect was noted as a result of reactivation by cysteine.

TABLE I

Effect of Metal Ions on the Ficin-catalyzed Synthesis of Hippurylanilide

The yield is given in per cent of the theoretical

		Yield of hippurylanilide									
Activator→		None (control)				Cysteine		KCN		Rongalit C	
Ficin→		Non-dialyzed		Dialyzed		Dialyzed		Dialyzed		Dialyzed	
Metal ion final concentration 10^{-n} *M* / Yield↘		%	Ratio	%	Ratio	%	Ratio	%	Ratio	%	Ratio
None (control)		52.2	100	53.0	100	62.2	100	62.5	100	53.0	100
Cu^{++}	2	4.0	8	4.7	9	0	0	3.2	5	1.9	3
	3	—	—	13.0	24	17.0	27	15.0	24	9.3	17
	4	—	—	26.6	50	35.9	58	43.3	69	45.0	85
Cd^{++}	2	28.0	54	23.8	45	32.3	52	29.0	46	27.5	52
	3	—	—	53.2	100	65.0	104	54.5	87	44.5	84
	4	—	—	52.2	98	71.0	114	65.5	105	48.5	91
Hg^{++}	2	3.3	6	2.8	5	13.0	24	1.3	2	2.0	3
	3	—	—	3.0	5	41.5	66	2.3	3	46.2	87
	4	—	—	27.6	52	64.0	103	40.0	64	54.7	103
Sn^{++}	2	—	—	57.0	107	—	—	—	—	—	—
	3	—	—	56.0	106	—	—	—	—	—	—
	4	—	—	53.2	100	—	—	—	—	—	—
Sn^{++++}	2	—	—	48.5	91	—	—	—	—	—	—
Mn^{++}	2	41.5	79	34.6	65	64.0	103	52.0	83	52.5	99
	3	—	—	42.7	80	—	—	—	—	—	—
	4	—	—	46.0	86	—	—	—	—	—	—
Fe^{++}	2	53.7	102	57.0	107	65.5	105	62.0	99	57.5	108
	3	—	—	54.0	102	—	—	—	—	—	—
	4	—	—	53.0	100	—	—	—	—	—	—
Fe^{+++}	2	49.0	93	48.3	91	62.7	101	55.0	88	49.0	92
	3	—	—	48.5	91	—	—	—	—	—	—
	4	—	—	51.0	96	—	—	—	—	—	—
Co^{++}	2	41.7	89	49.0	92	68.0	109	46.0	73	54.5	103
	4	—	—	53.0	100	—	—	—	—	—	—
Mg^{++}	2	56.2	107	54.0	102	66.5	107	65.4	105	53.0	100
	4	—	—	54.2	102	—	—	—	—	—	—
Al^{+++}	2	48.0	91	50.5	95	63.0	101	56.6	90	50.5	94
	3	—	—	49.5	93	—	—	—	—	—	—
	4	—	—	55.7	105	—	—	—	—	—	—

KCN-activated Enzyme—The addition of Cu^{++} and Cd^{++} showed inhibitory effects similar to those observed with cysteine-activated enzyme.

Hg^{++} exhibited such a strong inhibition as it did on the non-activated enzyme.

Rongalit C-activated Enzyme—The addition of 10^{-2}–10^{-3} M Cu^{++} had inhibitory effect on the hippurylanilide synthesis also in the presence of rongalit C as in the presence of other activators, but the inhibitory effect of 10^{-4} M Cu^{++} was greatly diminished by the addition of rongalit C. The effect of Cd^{++} in the presence of rongalit C was similar to that observed with the cysteine- or KCN-activated enzyme. At 10^{-2} M, Hg^{++} inhibited the action of ficin in the presence of rongalit C to nearly the same extent as it did in the absence of the activator. At 10^{-3}–10^{-4} M, however, Hg^{++} showed almost no inhibition as a result of the reactivation by rongalit C.

SUMMARY

1. The hippurylanilide-synthesizing activity of ficin is not changed by dialysis or by the addition of activators such as cysteine, potassium cyanide and rongalit C.

2. The addition of metal ions such as Cu^{++} and Hg^{++} exhibits a markedly inhibitory effect on the synthetic action of ficin in the absence of activator. The addition of Mn^{++} or Cd^{++} at a final concentration of 10^{-2} M is somewhat inhibitory. The other metal ions including those which were known to stimulate the papain-catalyzed synthesis have almost no influence on the synthetic reaction by ficin.

3. Even in the presence of cysteine, potassium cyanide and rongalit C, the synthetic activity of ficin is inhibited strongly by Cu^{++}, Hg^{++} and weakly by Cd^{++}.

The author wishes to express her deep gratitude to Prof. S. Utzino for his constant guidance.

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STUDIES ON THE NUCLEIC ACIDS FROM B. ALCALIGENES FAECALIS*

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Concerning the chemical nature of microbial nucleic acids an earlier study made by one of the authors (*1*) reported that *S. typhi murium* contained a kind of nucleic acid which the author considered to be a new type at that time. Since then especially in the last dacade the analytical techniques for biological materials including nucleic acids have made marvelous progress. The present research was attempted to renew study on this line with the nucleic acids isolated from *B. alcaligenes faecalis*, the lipids of which have already been investigated (*2*), by use of the techniques recently developed by several workers. It is hoped that this kind of work might be extended in the future to a variety of species of microorganisms characterized by different biological behaviors and also to those grown under various conditions.

EXPERIMENTAL

Isolation of Nucleic Acids from B. alcaligenes faecalis—The bacterial cells were grown in an ordinary broth for 5 days, collected and extracted with ethanol—ether (1:1) and then with chloroform. The defatted bacteria were ground thoroughly employing a stainless steel ball mill, extracted with a dilute acetic acid solution (pH 4.0) and then with a phosphate buffer (pH 6.6). The phosphate buffer extract was made to pH 2.6 with glacial acetic acid. The preicpitated nucleoproteins were dissolved in water with the addition of bicarbonate and reprecipitated with glacial acetic acid at pH 4.0. The latter precipitate was again dissolved in water and the solution adjusted to pH 6.0 was subjected to the protein-chloroform gel procedure by Sevag to be free of proteins. The protein free solution was concentrated, made to pH 7.4 with alkali, and dialyzed against distilled water and finally vacuum evaporated to dryness, obtaining a light brown powder in a yield of 1.2 g. from 100 g. of dry bacteria.

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* This study was presented before the Annual Meeting of the Japanese Biochemical Society held on April, 1955 in Kyoto.

The analytical data on this nucleic acid sample are as follows: N, 13.53 per cent (Dumas); P, 6.90 per cent (Allen); atomic N:P ratio 4.3; The ultraviolet absorption spectrum was measured with a sample dissolved in 0.01 *N* NaOH, giving ε (P) 8100 at $E_{max.}$ (261.5 mμ) and ε (P) 4700 at $E_{min.}$ (232.5 mμ).

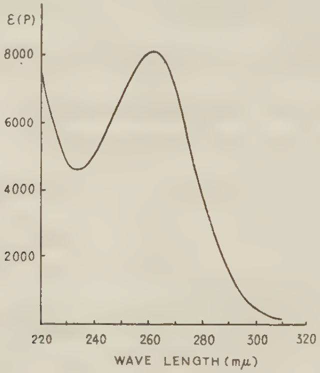

FIG. 1. Ultraviolet absorption spectrum of alcaligenes nucleic acids.

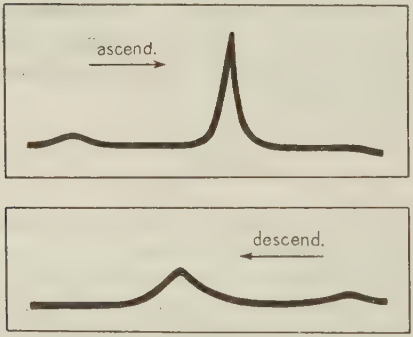

FIG. 2. Electrophoretic patterns of alcaligenes nucleic acids.
Sample, 0.5 per cent solution; Phosphate buffer, pH 7.4; Ionic strength 0.2; Temperature 8°; Specific conductivity 0.00209; Feild strength 30 volts; Current 0.1 mA./sq.mm.; Duration of electrophoresis 1 hour; Electrophoretic migration rate 18.6×10^{-5} cm²/volt. sec. (ascending), 18.0×10^{-5} cm²/volt. sec. (descending).

Electrophoretic Behavior—The nucleic acids were examined in a Tiselius apparatus as well as by paper electrophoresis with the results that in either case they moved as a single boundary as can be seen in Fig. 2 and Fig. 3.

Detection of Purines and Pyrimidines—3–6 mg. of sample were hydrolyzed in a sealed tube with 0.1 ml. of 70 per cent $HClO_4$ by heating at 100° for 2 hours according to the hydrolysis time recommended by Crosbie (*3*). After the completion of the hydrolysis, 0.16 ml. of 40 per cent NaOH were added by a pipette to neutralize about 3/4 the amount of $HClO_4$ present in the tube, followed by the addition of water to make the total volume 0.4 ml. 0.01 ml. of the well mixed solution was applied by a micropipette to a Toyo Paper No. 5A (3×45 cm.) at a point 5 cm. above the lower edge to form a circle of 1 cm. diameter, after which the wett spot was neutralized by being exposed to ammonia vapors and dried. The paper was subjected to the ascending chromatography which was carried out with 0.5 *N* ammonia-*n*-butanol (1:7) at 15° for about 15–20 hours. In conducting this chromatography sharp resolutions as well as good reproducibilities were obtained in such a manner that a small bunch of cotton wool made wet with 2 or 3 ml. of 10 per cent ammonia was fastened to the middle part of the vertical glass rod of the paper holder in order to secure the constancy of the vapor pressure of ammonia within the chromatograph cylinder. The developed chromatogram was air dried. The purine and pyrimidine bases were detected as dark spots on the paper by viewing the paper under ultraviolet light (Matsuda mercury lamp, 15 watts, 100 volts) through a 253.7 mμ short-wave filter prepared by Iwase Laboratory of the Scientific Research Institute, Ltd. in Tokyo. Detection limits for these bases were approximately 0.3 μg. per sq. cm. of paper area. Fig. 4 illustrates the paper chromatograms obtained with the hydrolysates of alcaligenes nucleic acids along with a series of reference samples.

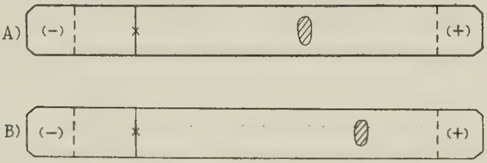

FIG. 3. Paper electrophoresis of the alcaligenes nucleic acids.
A): Borate buffer (pH 8.4, ionic strength 0.4).
B): Phosphate buffer (pH 8.0, ionic strength 0.2).
Paper, Toyo filter paper No. 51 (9×45 cm.); Field strength, 20 volts per cm.; Duration of electrophoresis, 3.0 hours; Operation at room temperature (15°); by the horizontal strip method.

The above data make it clear that the nucleic acids being studied contain all of the five nitrogenous bases, adenine, guanine, cytosine, uracil, and thymine, but no 5-methylcytosine.

Detection of Sugars—The present nucleic acids gave positive tests for the diphenylamine reaction as well as the orcinol-HCl reaction, indicating the presence of pentose as well as deoxypentose.

Experiment 1 (*Detection of* D-*Ribose*)—5 mg. of the nucleic acids together with 1 ml. of 1.0 *N* HCl were placed in a sealed tube and heated at 100° for 1 hour. 0.02 ml. of the hydrolysate were applied to Tôyô filter paper No. 51 (10×45 cm.) on a starting line 5 cm. above the lower edge. The paper was chromatographed by the ascending

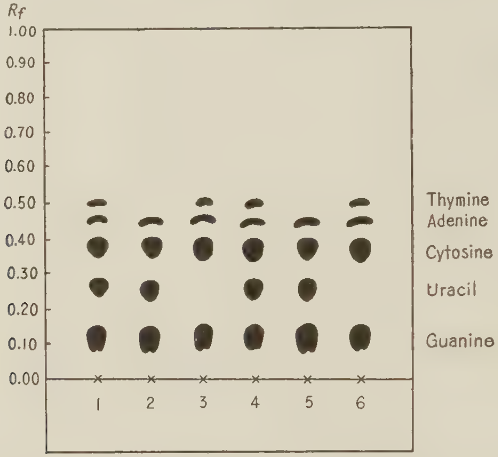

FIG. 4. Paper chromatograms of the hydrolysates of alcaligenes nucleic acids including a series of control chromatogram patterns.

Solvent: 0.5 *N* ammonia-*n*-butanol (1:7).

Samples:

1) Mixture of adenine, guanine, cytosine, uracil, and thymine from definite sources as given below.
2) Hydrolysate of yeast RNA.
3) ,, ,, thymus DNA.
4) Hydrolysate of alcaligenes nucleic acids.
5) ,, ,, ,, RNA.
6) ,, ,, ,, DNA.

Adenine ($E_{max.}$=262.5 mμ): Isolated from the acid hydrolysate of yeast RNA according to Levene. Guanine ($E_{max.}$=249 mμ): Isolated in the same way as above. Cytosine ($E_{max.}$=275 mμ): Synthesized by Hitching's method. Uracil ($E_{max.}$=259 mμ): Synthesized followingthe procedure by Davidson and Baudisch. (Thymine=$E_{max.}$) 264.5 mμ): Isolated from the hydrolysate of thymus DNA. The purity of these bases was checked by measuring their $E_{max.}$ as indicated in the parentheses.

techniqueusing two different solvent systems, a) *n*-butanol—acetic acid—water (4:1:5) and b) phenol—pH 9.2-borate buffer (8:2). After spraying the chromatogram with the aniline hydrogen phthalate reagent and heating it at 105° for 5 minutes, a single spot was observed having an R_f value identical with that of D-ribose.

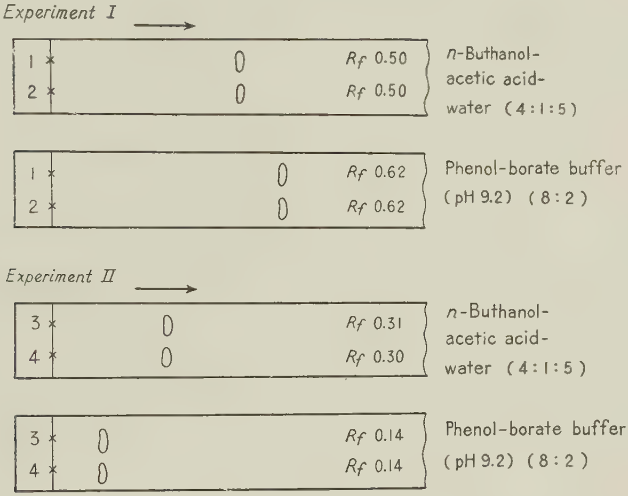
Experiment I
1
2
Rf 0.50
Rf 0.50
n-Buthanol-acetic acid-water (4:1:5)
1
2
Rf 0.62
Rf 0.62
Phenol-borate buffer (pH 9.2) (8:2)
Experiment II
3
4
Rf 0.31
Rf 0.30
n-Buthanol-acetic acid-water (4:1:5)
3
4
Rf 0.14
Rf 0.14
Phenol-borate buffer (pH 9.2) (8:2)

FIG. 5. Paper chromatograms of sugar components of alcaligenes nucleic acids.

Sample 1: D-Ribose (10 μg. per 0.01 ml.).
,, 2: Hydrolysate of alcaligenes nucleic acids (Hydrolysis with *N* HCl, 100°, 1 hour).
,, 3: Hydrolysate of the digests of standard DNA with DNase (Hydrolysis at pH 1.5 with dil. HCl, 100°, 12 minutes).
,, 4: Hydrolysate of the combined digests of alcaligenes nucleic acids with DNase and phosphatase (Hydrolysis at pH 1.5 with dil. HCl, 100°, 12 minutes).
Spray reagent: Aniline hydrogen phthalate.

Experiment 2 (*Detection of 2-Deoxy-*D*-ribose*)—40 mg. of the nucleic acids were taken in a small centrifuge tube, to which were added 4.0 ml. of DNase* solution containg 2 mg. enzyme per ml. measured by the Kunitz's densitometric method (*4*), 6.0 ml. of acetate buffer of pH 5.5 and 0.4 ml. of 0.05 *M* $MgSO_4$ and the entire mixture was thoroughly mixed and kept at 5–8° for 12 hours. A light brown precipitate of RNA, which formed when the digestion mixture was adjusted to pH 3.5, was removed by centrifugation. After deproteinization by the protein-chloroform gel method the protein free solution was brought to pH 1.5 with 1.0 N HCl and hydrolyzed by heating at 100° for 12 minutes. 0.02 ml. of the hydrolysate were examined by paper chromatography under the same conditions as described above, demonstrating a spot identical with 2-deoxy-D-ribose from thymus DNA. In Fig. 5 are shown the data from the above two experiments.

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* This enzyme was prepared from ox pancreas employing the technique by Kunitz.

Quantitative Estimation of DNA and RNA Contents in the Alcaligenes Nucleic Acids

DNA—Seibert's technique (*5*) based on the diphenylamine reaction was employed. 2 ml. of a sample solution containing 3.5–30 μg. DNA-P or 7–70 μg. DNA per ml. were mixed with 4 ml. of diphenylamine reagent which was prepared by dissolving 1 g. of pure diphenylamine in 100 ml. of glacial acetic acid (G. R.) and adding 2.75 ml. of concentrated H_2SO_4 (G. R.). The reaction mixture was heated at 100° for 10 minutes. A blank containing water instead of the unknown was run simultaneously. The DNA solution gave a blue color, the optical density of which was measured by a Beckman type spectrophotometer at the absorption maximum of 595 mμ. The observed DNA values needed a certain correction for two reasons. The first reason is that both the diphenylamine reaction and the orcinol-HCl reaction are given only by the sugars linked with purines and not by the sugars attached to the pyrimidines. The second reason is that the purine to pyrimidine ratio of the nucleic acids being studied was significantly different from that of the DNA used as standard. To meet this requirement the following correction formula was found satisfactory.

$$F=(Pu+Py)/2\,Pu$$

F: A factor by which any observed value is to be multiplied to obtain an actual value.

Pu: Purine content relative to pyrimidine content Py.

Example: If Pu/Py=1.20,
then F=0.917
therefore, actual DNA-P=0.917 × observed DNA-P.

RNA—The assay was carried out mainly according to the Kerr technique (*6*). In a test tube calibrated at 15 ml. were introduced 5 ml. of a sample solution containing 5–50 μg. of RNA-P or 10–100 μg. of RNA per ml., and 5 ml. of conc. HCl (G. R.) containing 0.02 per cent $FeCl_3 \cdot 6H_2O$ (G. R.), and 0.3 ml. of 10 per cent orcinol in ethanol. The resulting mixture was heated at 100° for 30 minutes to form a green colored solution, after which it was cooled under running tap water and made up with water to a total volume of 15 ml. A blank containing water instead of the sample solution was run simultaneously. The optical density of the colored solution was measured at the absorption maximum of 670 mμ.

In this case double corrections were necessary for obtaining a true value of RNA. The nucleic acids which contain significant amounts of DNA besides RNA respond to some extent to the orcinol-HCl reaction, resulting in the overestimation of RNA. The first correction for this error was made by subtracting 0.158 μg. RNA-P per 1 μg. of

DNA-P from the total RNA-P value found. This correcting factor was obtained following the correction formula introduced by Mejdaum (*7*). The second correction was based on the same reason as described previously in the DNA, therefore the first corrected value was multiplied by a calculated factor F.

The results thus obtained are illustrated in Table I.

From the above Table is evident that the combined yield of DNA-P and RNA-P in per cent with respect to total P is 100.1 per cent (15.6+84.5) when corrected or 108.3 per cent (16.4+91.9) when uncorrected.

TABLE I

DNA and RNA Contents in the Alcaligenes Nucleic Acids

a) DNA Contents (Diphenylamine reaction)

Sample amoun		DNA-P found		Yield of DNA-P in per cent with respect to total P	
As-Na-nucleate (μg.)	As nucleic acid-P (μg.)	Uncorrected (μg.)	Corrected (μg.)	Uncorrected	Corrected
304	21.0	3.45	3.28	16.4	15.6

b) RNA Contents (Orcinol—HCl reaction)

Sample amount		RNA-P found		Yield of RNA-P in per cent with respect to total P	
As-Na-nucleate (μg.)	As nucleic acid-P (μg.)	Uncorrected (μg.)	Corrected (μg.)	Uncorrected	Corrected
304	21.0	19.3	17.7	91.9	84.5

Quantitative Analyses for Purines and Pyrimidines of Alcaligenes Nucleic Acids

Preliminary Experiments to Purine and Pyrimidine Assay—Chromatograms were developed with given amounts of authentic specimens of purine and pyrimidine according to the manner described previously under the detection of these bases. The dried chromatograms were subjected to the extraction procedure for the individual spots as follows. For this purpose three sheets of chromatograms run simultaneously were cut at appropriate portions corresponding to the individual spots in an equal size and the combined three cuts were cut further into many small pieces and transferred into a glass-stoppered test tube. 5 ml. of appropriate eluting solvent were added and the tubes were placed in a closed vessel containing a little water for facilitating vapor equilibration within it and the vessel was kept at 37° for 15 hours, during which time the tube content was shaked vigorously several times for a few minutes to effect quantitative elution of materials. Eluting solvents employed were 0.1 *N* HCl for adenine, cytosine, uracil, and thymine, and 1.0 *N* HCl for guanine. The eluate was decanted into

TABLE II

Ultraviolet Absorption Data on Purines and Pyrimidines

Kind of Sample	Amount of Sample	Extinction*	Ext. coeff.** $E^{10\,\mu g}_{1\,cm.}$
Guanine in *N* HCl		$E_{249\,m\mu-290\,m\mu}$	
	10.87	0.097	0.475
	21.74	0.201	0.461
		average	0.468 (0.475^a)
Adenine in 0.1 *N* HCl		$E_{262.5\,m\mu-290\,m\mu}$	
	10.26	0.189	0.920
	20.52	0.383	0.932
		average	0.926 (0.935^b)
Cytosine in 0.1 *N* HCl		$E_{275\,m\mu-300\,m\mu}$	
	10.02	0.111	0.549
	20.04	0.215	0.549
		average	0.549 (0.546^c)
Uracil in 0.1 *N* HCl		$E_{259\,m\mu-280\,m\mu}$	
	9.96	0.121	0.608
	19.92	0.244	0.613
		average	0.611 (0.609^d)
Thymine in 0.1 *N* HCl		$E_{264.5\,m\mu-290\,m\mu}$	
	9.44	0.102	0.539
	18.88	0.205	0.540
		average	0.540 (0.545^e)

* All of the extinctions are average values of three experiments.

** Extinction coefficients in parenthesis: (a) and (e) were reported by Chargaff (*8*) and (b), (c), and (d) were given by Crosbie (*3*).

a centrifuge tube as much as possible and centrifuged to obtain a completely clear supernatant which was then used for extinction measurement in a 1 cm. cell of the Beckman type spectrophotometer (Hitachi EPB-U type). The extinctions of all eluates were determined by the difference method according to Vischer and Chargaff (*8*), *i.e.*, from the difference between both extinction readings, of which one was made at the absorption maximum and the other at a certain reference wavelength of low absorption. A plot of optical density against concentration with any purine or pyrimidine base yielded a definite reference curve for quantitative estimations for that base.

Table II presents the ultraviolet absorption data on purines and pyrimidines established in our laboratory, indicating a fair agreement with those values in parentheses found in the literature.

TABLE III

Purine and Pyrimidine Contents of the Alcaligenes Nucleic Acids and the Derived DNA and RNA Fractions Expressed in Terms of Molar Proportions per 100 g.-atoms of P in Hydrolysate Corrected for a 100 per cent Recovery

Purine and Pyrimidine	*B. alcaligenes*			Yeast RNA	Thymus DNA
	Nucleic Acids	DNA fraction	RNA fraction		
Adenine	22.7	19.3	24.8	23.5	21.2
Guanine	31.8	33.8	30.6	27.5	29.1
Cytosine	20.3	36.1	21.8	27.3	24.8
Uracil	20.6	—	22.7	21.7	—
Thymine	4.3	10.8	—	—	24.8
Actual recovery	88.7	97.2	86.7	96.4	98.6
Pu:Py	1.21	1.13	1.24	1.04	1.01
AT:GC	0.52	0.43	—	—	1.16
AU:GC	0.84	—	0.91	0.82	—
A:T	5.2	1.77	—	—	0.85
G:C	1.55	0.93	1.40	1.00	1.17

Assay for the Purine and Pyrimidine Contents in the Alcaligenes Nucleic Acids and the Derived DNA and RNA Fractions—The alcaligenes nucleic acids as well as the DNA and RNA fractions derived from the former by the technique of Thannhauser—Schmidt were analyzed for their purine and pyrimidine contents in the same way as the above procedure. DNA measurement by the diphenylamine reaction indicated that the RNA fraction contained 2 per cent of DNA. This amount of contamination, however, was so small that it did not seemed to lead to any significant error in the purine and pyrimidine assay for the RNA fraction. From the observed values various ratios of the base components, such as purine: pyrimidine, AT:GC, AU: GC, A:T, and G:C were calculated. For comparative purpose the same analyses were conducted simultaneously with specimens of the yeast RNA and the thymus DNA. The whole experimental data are summarized in Table III.

DISCUSSION AND RESULTS

Nucleic acids were prepared under relatively mild conditions from *B. alcaligenes faecalis* cultured for 5 days and they proved to be electrophoretically homogeneous when tested by a Tiselius apparatus. That they contain as sugar components D-ribose and 2-deoxy-D-ribose and as purine and pyrimidine bases adenine, guanine, cytosine, uracil, and thymine was demonstrated by paper chromatography employing authentic specimens as control. Quantitative estimation of these sugar components as well as nitrogenous components has led to the results that the nucleic acids consists of both types of RNA and DNA in an approximate proportion of 85 :15. In evaluating the contents of RNA and DNA in the nucleic acids being studied, certain corrections were found necessary for the reasons mentioned above. By employing the correction formula presented a fair total recovery of DNA-P and RNA-P was obtained, otherwise the overestimation of their values was inevitable.

The nucleic acids were separated into two fractions DNA and RNA. These three kinds of nucleic acids, *i.e.*, the original nucleic acids, and the derived DNA and RNA fractions were subjected to the quantitative analysis for their base components. As previously mentioned in Table III, all of the alcaligenes nucleic acids examined showed higher purine to pyrimidine ratios ranging from 1.13 to 1.24, while the yeast RNA and the thymus DNA, both of which were simultaneously analyzed as control, gave values almost constant within 1.04–1.01. From the data presented herein it may be apparent that the alcaligenes DNA fraction is characterized by an appreciably high content of guanine and cytosine and a considerably low content of thymine, thus showing a low AT : GC ratio of 0.43 and a high A :T ratio of 5.2.

With regard to the base composition of DNA of microorganism, Chargaff is of the opinion that the microbial nucleic acids may be grouped into three principal families on a basis of the molar ratio of purine to pyrimidine (AT :GC), namely, a) AT-type rich in adenine and thymine, b) GC-type of high guanine and cytosine contents, c) AT-GC type, an intermediate type containing those four bases in nearly equal quantities. From his review made on this subject (9) the following examples may be cited, in which the figure in parenthesis indicates the value of molar raito involved in the grouping.

AT-type: *Yeast* (1.80)- *Pneumococcus type III* (1.59)- *Hemophilus influenzae* (1.64)

AT-GC-type (intermediate type): *E. coli mutant* (0.99)- *E. coli K-12* (1.00)- *E. coli thymineless* (1.01)

GC-type: *Mb. tuberculosis human* (0.58)- *Mb. tuberculosis bovine* (0.58)- *Mb. tuberculosis avian* (0.42)- *Mb. phlei* (0.50)

In view of the above criteria of classification the alcaligenes DNA may be grouped into the GC-type, but as an atypical member charactized by exceedingly high contents of guanine and cytosine and an exceptionally low content of thymine.

On the other hand the alcaligenes RNA fraction which was analyzed demonstrated that it was rich in purines especially guanine giving a purine: pyrimidine ratio of 1.24 and possessed cytosine and uracil in almost equimolecular quantities. Concerning the microbial RNA, only few studies have been made to date. According to the reports available guanine appears rather to predominate in most of the RNA from microorganisms including yeast and there is usually found the purine: pyrimidine ratio exceeding unity in the range from 1.1 to 2.1.

Now here is one problem open to question. In cells of organisms, as is generally believed, there should occur quite a number of DNA and RNA which differ from each other in physical, chemical, and biological properties and also vary in distribution within the cells. According to the work on nucleic acids made by Deimel and Maurer (*10*), it has been demonstrated that the nucleic acids prepared from the liver and other organs of rats are separable into DNA and RNA by paper electrophoresis, in which P^{32} was used as tracer for locating the nucleic acids on the chromatograms. The present research has evidenced that the nucleic acids isolated from *B. alcaligenes* are electrophoretically homogeneous even though they are composed of DNA and RNA types of nucleic acids. It may be possible to assume that the originally prepared nucleic acids should be an admixture of various RNA and DNA and these nucleic acids might have happened to combine by some certain binding forces during the procedures of preparation, thus causing them to behave apparently as a unit body in the electric field. At the present stage of the investigation, however, no substantial evidence for this assumption is available.

SUMMARY

1. Nucleic acids were prepared from the nucleoproteins isolated from a certain strain of *B. alcaligenes faecalis* and they proved to be electrophoretically homogeneous.

2. The preparation contained RNA and DNA in an approximate proportion of 85 :15 and these two component nucleic acids were fractionated and analyzed.

3. The DNA represented an atypical GC-type which was quite rich in guanine and cytosine and was exceptionally poor in thymine. The RNA was shown to be high in purine content especially guanine, having a purine : pyrimidine ratio of 1.24.

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HOMOGENTISIC ACID AS A GROWTH FACTOR OF BAMBOO SHOOT

Sirs:

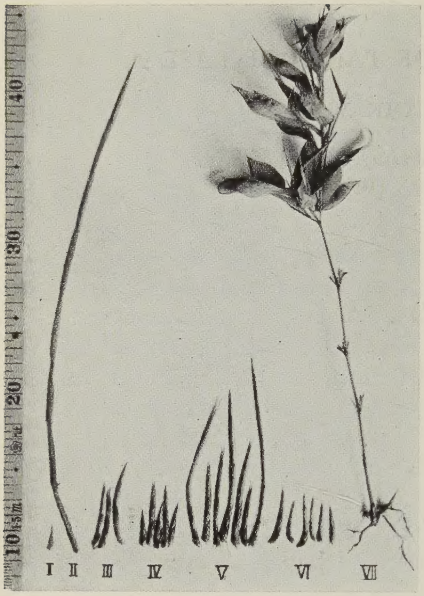
A characteristic taste of bamboo shoot, called *egomi* or *egumi* in Japanese, which irritates the tangue root has recently been investigated by Hasegawa in this laboratory (*1*). A similar taste is also recognized in *taro* and *hange* (*Pinellia ternata Tenure*, Chinese drug) *etc.* Bamboo shoot contains tyrosine at high concentrations, which is often found in crystalline mass in the canned bamboo shoot. Therefore the compounds responsible for *egomi* in bamboo shoot were searched for among tyrosine derivatives, and homogentisic acid was shown to be one of them at least. The finding was strengthened by the fact that crystalline homogentisic acid was obtained from bamboo shoot, and the presence of this compound was also demonstrated paperchromatographically in *taro* and *hange*.

Indoleacetic acid (heteroauxin) is a well-known growth factor of plants, and α-naphthylacetic acid has also the same effect (*2*). Since the growth of the bamboo shoot is very rapid, the presence of homogentisic acid (2,5-dihydroxyphenylacetic acid) in it led the authors to make the following experiment with bamboo shoot.

The bamboos (*Schibataea Kumasasa*, *Makino*), which had already shoots under the earth, were digged, and planted in four pots filled with washed sand. The following four kinds of solution were poured into the pot respectively every morning from May 26th to June 8th.

As shown in the photograph, only homogentisic acid showed a high activity in stimulating the growth of the bamboo shoot, compared with other compounds.

Even the growth of the group supplemented with homogentisic acid was much less than that grown on earth. Last year a similar experiment was carried out with the bamboo shoots growing on earth. However, their growth were too rapid to be distinguished from each other, suggesting that the experiment is preferable to be performed under the malnutritional condition.



I=26/May; II=on earth; III=a; IV=b; V=c; VI=d; VII=adult.

a. 1 per cent glutamic acid monosodium 20 ml.
water 20 ml.

b. 40 mg. per cent tyrosine 20 ml.
1 per cent glutamic acid monosodium 20 ml.

c. 40 mg. per cent homogentisic acid 20 ml.
1 per cent glutamic acid monosodium 20 ml.

d. 40 mg. per cent 2,5-dimethoxy phenylacetic acid 20 ml.
1 per cent glutamic acid monosodium 20 ml.

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